ENDOGENOUS AMINO ACID TRANSPORT AND TRANSLATION OF RAT LIVER mRNA IN *XENOPUS LAEVIS* OOCYTES

by

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To Pam and Darwin.

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Neutral and cationic amino acid transport was studied in stage 6 Xenopus laevis oocytes and found to be mediated by Na^+-dependent and Na^+-independent processes. Using the criteria of cis- and trans-inhibition, pH sensitivity, and stereoselectivity, several distinct transport activities were identified. The characteristics of some of these activities resembled those of transport systems previously described in other tissues. Evidence is presented which supports the presence of activities similar in many respects to Systems ASC, asc, y^+, L, and $B^{0,+}$. Although a portion of the transport of 2-aminoisobutyric acid (AIB) into oocytes was found to be Na^+-dependent, the data do not support mediation by System A.

The effect of meiotic maturation, also referred to as germinal vesicle breakdown (GVBD), on the transport of AIB,

threonine, and leucine in oocytes was also investigated. The *in vitro* induction of GVBD was achieved using progesterone or tetradecanoylphorbol acetate (TPA). Following the induction of GVBD, the Na^+-dependent and Na^+-independent transport rates of all substrates tested declined markedly.

The results of experiments investigating the oocyte-mediated synthesis of liver phosphoenolpyruvate carboxykinase (PEPCK) and serum albumin (RSA) following microinjection of rat liver mRNA are also reported. The quantities of PEPCK and RSA were found to increase with time up to about 48 hours after microinjection. Further incubation resulted in a decline in the amount of the two proteins. In addition, the microinjection of 20 ng or less of mRNA per oocyte was found to be more efficient than the microinjection of quantities greater than 20 ng. Furthermore, the oocyte-mediated translation of mRNA extracted from the livers of fed, glucagon-treated, or diabetic rats was shown to reflect the relative quantities of PEPCK and RSA mRNA present as revealed by Northern analysis. Experiments involving the microinjection and re-extraction of mRNA revealed that the transcripts coding for the two proteins are quite stable in the oocyte. The mRNAs coding for both PEPCK and RSA were estimated to have half-lives of at least three days in the oocyte.

CHAPTER I

INTRODUCTION

The South African clawed frog, Xenopus laevis, was first described in 1803 by F.M. Daudin. Originally believed to belong to the genus Bufo, it was not classified as a member of the family Pipidae until many years later and its present Latin name not officially adopted until 1890 (Leslie, 1890). The skin of the animal is smooth and the body flattened dorsoventrally. The fore and hind limbs are splayed out to the sides and, while in an ideal posture for swimming, do not effectively support terrestrial locomotion. The claws of Xenopus laevis, located on the hind feet, are used to help tear food into manageable chunks. Breeding takes place during the rainy season at a time when the ponds contain ample water to support the growth of the larvae. The female is capable of ovulating from 500 to 1,000 eggs in a single 24-hour spawning period. Fertilization occurs in the water and metamorphosis takes approximately 2 months to complete (Deuchar, 1975). The animals do well in captivity and can remain healthy for many years on a diet of vertebrate heart or liver. Unlike Rana pipiens, the ovary of the captive Xenopus female contains oocytes at all stages of maturation. The oocytes

are easily obtained from the anesthetized animal and can survive many days in a comparatively simple buffered saline solution supplemented with essential ions and antibiotics (Wallace et al., 1973). The impressive size of the fully matured oocyte, over 1 mm in diameter, facilitates manipulation. For these reasons, the African clawed frog has become an important tool in biochemical and biological research.

The use of Xenopus laevis oocytes as a means of carrying out the translation of exogenous mRNA began in 1971 in the laboratories of Gurdon and Marbaix (Gurdon et al., 1971; Lane et al., 1971). In these series of experiments, the co-injection of purified haemin and rabbit reticulocyte 9S RNA was shown to bring about the synthesis in oocytes of a hemoglobin-like moiety that was chromatographically indistinguishable from the authentic molecule isolated from rabbits. Soon after this landmark work, further reports of the oocyte-mediated translation of foreign mRNA began appearing in the literature. Examples of the translation of mRNA isolated from vertebrates, (Vassart et al., 1975; Lane and Knowland, 1975; Reynolds et al., 1975), invertebrates (Lane et al., 1983), viruses (DeRobertis et al., 1977), and plants (Lane et al., 1981; Matthews et al., 1981) clearly demonstrated the flexibility and fidelity of the Xenopus oocyte system.

Although the translation of heterologous mRNA in Xenopus oocytes has been used effectively in diverse spheres of research, it has been particularly beneficial to the study of integral membrane proteins, such as hormone receptors (Bahouth et al., 1988), ion channels (Leonard et al., 1987), and nutrient transporters (Hediger et al., 1987a,b). Unlike cytoplasmic enzymes, whose presence can often be followed easily through a purification scheme by means of appropriate activity assays, channels and transporters generally must be situated at a hydrophobic interface separating 2 hydrophilic compartments in order to be detected. Although the reconstitution of solubilized membrane proteins into artificial proteoliposomes accomplishes this partitioning (Hirata, 1986; Maloney and Ambudkar, 1989), many channels and transporters do not possess the stability necessary to survive rigorous solubilization procedures. Translation of foreign mRNA in oocytes, however, results in the correct positioning of functional integral membrane proteins into the oocyte plasma membrane (Sumikawa et al., 1981; Barnard et al., 1982; Parker et al., 1985). Further, purification strategies can be designed which focus on the identification of the mRNA, or corresponding cDNA, coding for a particular protein rather than on the protein itself (Hirono et al., 1985). The use of the Xenopus oocyte in this role, that is, as a screening device for the

characterization of specific cloned cDNAs, is exemplified by the work of Wright and his co-workers with the cloning of the intestinal Na^+-dependent glucose transporter (Hediger et al., 1987a,b). Lacking an antibody directed against the protein, the authors identified a cDNA coding for the glucose carrier by means of mRNA translation in Xenopus oocytes. Initial experiments involving the microinjection of rabbit intestinal poly(A)$^+$ mRNA into oocytes resulted in an 8-fold increase in the Na^+-dependent transport of methyl-α-D-glucopyranoside (MeGlc). Following successive rounds of mRNA size fractionation via agarose gel electrophoresis and microinjection, a class of mRNA averaging 2.3 kb in length was identified that increased the rate of Na^+-dependent MeGlc transport into oocytes by 10-fold over that seen with adjacent size fractions (Hediger et al., 1987b). The active fraction was used to prepare a cDNA library using an in vitro expression vector (Hediger et al., 1987a). Synthetic RNAs transcribed from increasingly smaller pools of clones were microinjected into oocytes which were then assayed for Na^+-dependent MeGlc transport. The single clone that was ultimately identified boosted MeGlc transport by more than 1,000-fold over controls.

This manuscript, in essence, describes endogenous amino acid transport in Xenopus oocytes and the development of methodology to study the oocyte-mediated synthesis of

hepatic proteins following the microinjection of rat liver mRNA. Although the bulk of the experiments which are described were conducted with the eventual expression of the rat liver System A amino acid transporter in mind, the approach should be applicable to all plasma membrane transporters.

Prior to studies involving the investigation of specific heterologous transport or channel activities in Xenopus oocytes, it is necessary to first verify the level of expression in the uninjected oocyte of the activity in question. For example, before undertaking experiments involving the microinjection of rabbit intestinal mRNA, Hediger et al. (1987b) initially characterized the endogenous glucose transport activities in the oocyte. The results of these preliminary investigations revealed the presence of a transport activity resembling the facilitated glucose transporter by virtue of its sodium independence. A Na^+/glucose co-transport activity, however, appeared to be absent from the uninjected oocyte (Hediger et al., 1987b). Given this low background level of endogenous activity, an increase in the Na^+-dependent transport of glucose resulting from the microinjection of mRNA, should be relatively easy to detect. As described above, such was the case for this particular transporter. In a similar manner, Houamed et al. (1984) have described the expression in oocytes of functional receptors for γ-aminobutyric acid,

glycine, and glutamic acid following the microinjection of rat brain mRNA. Earlier experiments had failed to detect a response in uninjected oocytes after the application of these amino acids (Kusano et al., 1982; Houamed et al., 1984). Likewise, investigations into the subunit structures of fetal and adult acetylcholine receptors were possible because of the absence of native receptors of this type in the oocyte (Kusano et al., 1982; Mishina et al., 1986). With these examples in mind, it is apparent that an investigation involving the mRNA-induced expression in oocytes of an amino acid transporter must begin with the characterization of endogenous transport activities. Chapters II and III of this manuscript describe such an investigation. Chapter II concerns the transport properties of 13 naturally-occurring and synthetic amino acids in the prophase-arrested oocyte. Chapter III, on the other hand, deals with alterations in amino acid transport observed following the in vitro induction of meiotic maturation. The investigation described in Chapter III is limited to 4 distinct transport activities in the oocyte which correspond in many ways to transport systems previously characterized in other species.

Chapter IV of this manuscript focuses on events taking place within the oocyte subsequent to the microinjection of rat liver mRNA. Based on experiments involving the microinjection of globin mRNA (Gurdon et al., 1971), it is

often assumed that foreign mRNA molecules are relatively resistant to degradation once inside the oocyte. However, globin mRNA, as well as the mRNAs coding for most other proteins studied by means of the oocyte system (Berns et al., 1972; Barnard et al., 1982), is particularly long-lived in vivo. The fate of mRNAs with short half-lives, however, such as that coding for the System A amino acid transporter, has not been investigated in the oocyte. Furthermore, although the majority of investigations referenced in this manuscript utilize post-microinjection incubations of 2 or 3 days, the optimum incubation time could vary greatly depending on the particular protein under investigation. In addition, the observation of literally hundreds of oocytes has demonstrated that the viability of the cell deteriorates after microinjection and that this deterioration is dependent, in part, upon the quantity and type of mRNA injected. Extension of the post-microinjection incubation period beyond the point where viability begins to decline would serve little purpose.

Due to the fact that the System A carrier has yet to be isolated in pure form, neither an antibody nor a cDNA corresponding to this transporter is available. Thus, the direct monitoring of the System A protein or its mRNA in the oocyte is not possible. For this reason, 2 other proteins, phosphoenolpyruvate carboxykinase (PEPCK) and rat serum albumin (RSA), were chosen to serve as models.

Following the microinjection of rat liver mRNA into oocytes, the oocyte-mediated synthesis of PEPCK and RSA was monitored via immunoblot analysis and the stability of the corresponding mRNAs by means of Northern analysis. Experiments are also described which examine differences in the quantities of PEPCK and RSA synthesized in the oocyte resulting from the microinjection of varying amounts of mRNA. In addition, a novel utilization of the oocyte mRNA translation system, that is, as a means of estimating relative quantities of specific mRNAs, is also described in this chapter. Following the microinjection of RNA extracted from the livers of rats under 3 conditions (fed, diabetic, or glucagon-injected), proteins are synthesized in the oocyte in quantities reflecting, in general, the abundance of their corresponding mRNAs in the liver donor.

CHAPTER II

CHARACTERIZATION OF NEUTRAL AND CATIONIC AMINO ACID TRANSPORT IN *XENOPUS LAEVIS* OOCYTES

Introduction

Amphibian oocytes have been used extensively in the study of numerous and diverse aspects of metabolism as well as cell and developmental biology. Because of their large size and relative ease of handling, oocytes from the African clawed frog, *Xenopus laevis*, are used routinely for investigations involving transcriptional and translational regulation, protein processing and targeting, and intracellular signalling (reviewed in Huez and Marbaix, 1986). Furthermore, isolated *Xenopus* oocytes have been employed extensively as an *in vivo* translation system for mRNA from other cell types (Colman, 1984; Huez and Marbaix, 1986). These studies have allowed the detailed investigation of a number of membrane-bound receptors (Williams *et al.*, 1988; Kumar *et al.*, 1988), ion-channels (Hirono *et al.*, 1985; Sigel, 1987), and the intestinal Na^+-dependent transport system for glucose (Hediger *et al.*, 1987a,b). Recently, Aoshima *et al.* (1988) have reported increased amino acid uptake in oocytes following injection of mRNA isolated from rat intestine. Prior to undertaking studies to test for expression of specific amino acid transport systems in

oocytes following microinjection of exogenous mRNA, it is helpful to have some knowledge of the characteristics of amino acid transport in the "basal" state, i.e., the prophase-arrested oocyte. For this reason, an investigation of the endogenous amino acid carriers in Xenopus oocytes was undertaken.

Transport of amino acids by oocytes has been reviewed recently (Van Winkle, 1988). Previous reports on alanine (Jung and Richter, 1983; Jung et al., 1984) and leucine (Belle' et al., 1976) transport into Xenopus oocytes have revealed that both amino acids are taken up from the surrounding medium in a saturable, Na^+-dependent fashion. Jung et al. (1984) concluded that a transport system analogous to the System A transporter exists in the oocyte and mediates Na^+-dependent alanine uptake. However, their studies also demonstrated that the Na^+-dependent alanine transport was not inhibited by an excess of the System A-specific amino acid analog 2-(methylamino)-isobutyric acid (MeAIB). Bravo et al. (1976) have examined the transport of several amino acids into oocytes. For example, alanine transport was shown to be inhibitable by the neutral amino acids glycine, valine, and leucine, but not by the charged amino acids arginine and glutamic acid. These data suggest the presence of a neutral amino acid carrier with broad substrate specificity, perhaps similar to System ASC (Kilberg, 1982). With regard to charged amino acids,

competition for transport between glutamic acid and aspartic acid, and between lysine and arginine was observed (Bravo et al., 1976). However, apart from proposing that oocytes may have separate transporters for the various classes of amino acids, no attempt was made by Bravo et al. (1976) to identify further the specific agencies responsible for the observed transport.

This chapter is an investigation of amino acid transport in prophase-arrested oocytes from X. laevis with emphasis on the identification and partial characterization of the agencies responsible for the transport of neutral and cationic substrates. Although the unambiguous verification of individual transporters must await protein identification, the utilization of substrate specificity, trans-effects, and inhibition analysis has made possible the preliminary identification of distinct carrier processes as defined previously in other cell types.

Materials and Methods

Isolation of Oocytes

Mature female Xenopus laevis (Xenopus I, Ann Arbor, Michigan) were housed in an aquarium containing synthetic pond water (1.4 mM NaCl, 0.05 mM $NaHCO_3$, 0.03 mM KCl, 0.003 mM Na_2HPO_4) at 18 to 20°C. The animals were fed either bovine liver or heart 3 times per week.

Ovarian tissue was removed from hypothermically-anesthetized animals and immediately placed into amphibian

Ringer's solution (115 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.6) at room temperature. The tissue was cut into smaller pieces of approximately 20-50 oocytes each and rinsed several times with fresh Ringer's solution followed by 2 rinses with modified Barth's medium (MBM) containing 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 10 μg/ml penicillin, 10 μg/ml streptomycin sulfate, 15 mM HEPES, pH 7.6 (Colman, 1984). Oocytes were manually separated from surrounding membranes (defolliculated) with Dumont #5 forceps and placed into fresh MBM at 18 to 20°C. Prior to experimentation, defolliculated oocytes were incubated overnight to facilitate recognition and removal of those damaged during the denuding process. Other studies have employed oocytes still surrounded by follicle cells ("follicles") or oocytes defolliculated by treatment with collagenase. We have determined that enzymatic isolation of oocytes with collagenase can cause loss of endogenous transport activity which is not regained upon incubation in vitro. As a result, all the oocytes used in the present work were isolated by hand defolliculation.

Amino Acid Transport

All transport assays described below were carried out using uptake tubes made from 5 ml polycarbonate centrifuge tubes. The conical end of the tube was cut off and a small piece of nylon mesh (Tetko Inc. No. HC-3-75, 75 μM mesh) was glued to the opposite end using plastic model cement. Excess

nylon mesh was trimmed from around the tube after the glue had dried thoroughly. All incubations and washes, described below, were carried out with the oocytes inside the uptake tubes. Oocytes were transferred by means of glass pipettes (1.5 mm inside diameter).

Prior to measurement of amino acid uptake, 10-12 oocytes were incubated twice for 10 min each in 15 ml of Na^+-free MBM. The Na^+-free MBM was made by substituting choline chloride and choline bicarbonate for the corresponding sodium salts. After the second incubation, the oocytes were transferred to 0.5 ml of uptake buffer, consisting of either Na^+-containing or Na^+-free MBM with 50 μM of ^3H-labelled amino acid. Following a 15 to 60 min incubation at room temperature, the oocytes were washed free of external isotope with 10 rinses of 4 ml each of ice-cold amphibian Ringer's and single cells were placed into individual scintillation mini-vials. Solubilization was achieved by incubating each cell in 200 μl of 0.2 N NaOH containing 0.2% sodium dodecyl sulfate. The amount of time required for complete solubilization varied depending on whether or not the vials were agitated throughout the incubation period. Vials vigorously agitated on an orbital shaker (American Rotator V, approximately 170 rpm) required only 3 hours while unagitated vials were normally incubated overnight. Following solubilization, the trapped radioactivity was quantitated via liquid scintillation spectrophotometry. Neutralization

of the solubilization buffer was not required with the scintillation cocktail utilized (3a70B, Research Products International Corp.).

The rate of Na^+-dependent transport was calculated by subtracting the rate observed in the absence of sodium from that observed in the presence of sodium. For cis-inhibition experiments, 5 mM of unlabeled amino acid were included in the uptake mixture. Unlabeled amino acids that are transported by the same carrier as the radioactively-labeled substrate will, in effect, cause a decrease in the net uptake of the substrate under scrutiny. Unless specified otherwise, all amino acids and analogs were the L-isomer. Where necessary, uptake mixtures were balanced with regard to osmolarity using choline chloride.

Given the long duration of the uptake assay, the question of metabolism of the substrate molecule by the oocyte was addressed. The degree to which radioactive substrate was incorporated into TCA-precipitable material was determined by first homogenizing oocytes after transport in buffer containing 100 mM NaCl, 1 mM phenylmethyl-sulfonylfluoride, 1% (v/v) Triton X-100, and 20 mM Tris, pH 7.6 (oocyte homogenization buffer, OHB). A 5 to 10 μl aliquot of the homogenate supernatant fraction was then added to 1 ml of 1 N NaOH, 1.5% (v/v) H_2O_2 in a plastic 15 ml conical tube and vortexed briefly. The tube was capped tightly and placed into a 37°C water bath. This incubation in alkali

serves to degrade any tRNA that may be charged with radioactive amino acid substrate. After 10 minutes, the tube was removed from the bath and 4 ml ice-cold 24% (w/v) TCA containing 2% (w/v) casein hydrolysate added and the mixture vortexed briefly. The tube was then cooled on ice for at least 30 minutes prior to filtration, with suction, through a glass fiber filter disk (Whatman GF/C). The tube was rinsed with 5 ml ice-cold 8% (w/v) TCA and the rinse poured over the filter. The filter was further washed with an additional 5 ml of 8% (w/v) TCA followed by 10 ml acetone. The filter was left under vacuum until dry and the radioactivity adhering to it quantitated via liquid scintillation spectrophotometry. The incorporation of histidine, alanine, threonine, serine, or cysteine into precipitable material was monitored for 60 min and shown to be less than 3.5% of the total amino acid accumulated. Even after a 24 h incubation the incorporation of the amino acids tested was less than 10%. For AIB and MeAIB there was no detectable incorporation into protein.

Metabolism of substrate molecules was further examined by means of paper chromatography. Following transport, oocytes were homogenized in OHB as described previously and 100 μl of the supernatant fraction added to 300 μl of absolute ethanol in a 1.5 ml Eppendorf microcentrifuge tube. The tube was placed at -20°C for at least 1 hour after which time it was centrifuged at 12,000 x g at 4°C for 30 minutes. The supernatant fraction was transferred to a clean tube and

centrifuged as above but only for 15 minutes. The supernatant fraction was again transferred to a clean tube and evaporated to dryness under vacuum. The residue was solubilized in 20 μl distilled, deionized water (ddH_2O) and 5 μl was used to determine total radioactivity via liquid scintillation spectrophotometry. Depending on the quantity of radioactivity present, 5 to 10 μl of the extract (i.e. the resolubilized residue) was spotted onto Whatman 3MM chromatography paper. Adjacent lanes contained unlabelled amino acid as well as pure radioactively-labelled amino acid. The chromatography paper was rolled into a tube shape, fastened with a staple, and placed upright in a glass chromatography tank containing approximately 150 ml N-butanol, acetic acid, and ddH_2O in the ratio of 12:3:5. The separation was allowed to proceed until the solvent front had migrated approximately 12 cm from the origin. The chromatography paper was removed from the tank, laid flat, and the solvent front marked with a pencil. The paper was allowed to air dry in a fume hood and individual lanes were then cut from the paper. Lanes containing unlabelled amino acid were sprayed with a 3% (w/v) solution of ninhydrin in 95% (v/v) ethanol, allowed to dry, and then heated in a drying oven at 100°C for 10 to 15 minutes or until color appeared. Heating the paper with a hand-held hair drier was also an effective means of catalyzing the ninhydrin reaction. Lanes containing radioactively-labelled amino acid or oocyte

extract were cut transversely into 1 cm strips and the radioactivity quantitated via liquid scintillation spectrophotometry. The calculation of R_f values was carried out by dividing the distance from the origin that the amino acid had migrated, as evidenced by the ninhydrin-positive spot or radioactivity peak, by that of the solvent front.

Results

In the animal cells studied to date, the Na^+-dependent transport of neutral amino acids is mediated principally by two distinct carriers, Systems ASC (Christensen et al., 1965) and A (Oxender and Christensen, 1963). The substrate specificity for System ASC is broad with some affinity for all neutral amino acids, but preference is shown for those with a sulfhydryl or hydroxyl group as typified by serine, threonine, or cysteine. In contrast, System A is characterized by a high sensitivity to H^+, trans-inhibition, and tolerance for some, but not all, N-monomethylated substrates (Christensen et al., 1965; Christensen, 1984; Kilberg et al., 1985). The latter characteristic is generally considered to be the most useful defining property to distinguish System A mediation from that of System ASC (Oxender and Christensen, 1963; Christensen, 1984). Historically, the non-metabolizable alanine analogs AIB (2-aminoisobutyric acid) and MeAIB have been the substrates of choice because their Na^+-dependent transport is nearly restricted to System A in a wide variety of cell types.

However, a portion of Na^+-dependent AIB uptake is mediated by System ASC in some cells (Kilberg et al., 1981).

The results of initial experiments investigating the transport into oocytes of several neutral and cationic amino acids are shown in Table 2-1. The highest rates of substrate transport in the presence of sodium were seen with cysteine (98.6 ± 7.4 $pmol \cdot oocyte^{-1} \cdot h^{-1}$), alanine (73.4 ± 4.4 $pmol \cdot oocyte^{-1} \cdot h^{-1}$) and leucine (62.5 ± 4.1 $pmol \cdot oocyte^{-1} \cdot h^{-1}$). As described in the Materials and Methods section, the portion of the total substrate transport that is dependent upon the presence of alkali ions in the medium can be estimated by performing the transport assay in the presence and absence of sodium or lithium, and then calculating the difference between the observed velocities. The amino acids for which uptake exhibited, proportionally, the greatest amount of sodium dependence were glycine and threonine. For both of these substrates, approximately 80% of the total transport was eliminated when sodium was replaced by choline in the uptake buffer. Conversely, approximately 93% of the total oocyte uptake of tryptophan occurred in the absence of sodium. Although most of the amino acids tested exhibited very little or no Li^+ co-transport, 3 amino acids, glycine, serine, and leucine, were shown to have Li^+-dependent transport velocities which were at least 30% of the Na^+-dependent value (Table 2-1).

Table 2-1. Transport of neutral and cationic amino acids into oocytes in the presence of Na^+, Li^+, or choline.

Substrate	Na^+	Li^+	choline
Ala	73.4 ± 4.4	34.2 ± 3.2	38.9 ± 3.2
Gly	8.9 ± 0.6	6.4 ± 0.7	1.8 ± 0.3
Gln	31.5 ± 3.6	13.8 ± 1.8	13.6 ± 1.8
Ser	46.0 ± 5.1	39.1 ± 3.6	30.2 ± 3.3
Thr	48.6 ± 2.9	11.8 ± 1.8	9.9 ± 2.4
CysH	98.6 ± 7.4	52.5 ± 6.0	44.3 ± 4.2
Arg	44.7 ± 3.5	32.0 ± 3.0	29.4 ± 5.4
His	39.2 ± 3.2	9.3 ± 1.9	23.4 ± 1.2
Leu	62.5 ± 4.1	38.7 ± 3.1	27.8 ± 3.2
Phe	43.0 ± 2.8	18.9 ± 3.0	21.5 ± 2.2
Trp	18.3 ± 0.9	11.4 ± 0.6	17.1 ± 1.1
AIB	10.4 ± 1.6	5.2 ± 0.5	4.1 ± 0.2

The oocyte transport of the amino acids listed above was assayed at 20°C for 15 or 60 minutes as described in the Materials and Methods section. The concentration of the substrate amino acids was 50 μM in all cases. Uptake mixes were made using MBM (Na^+ condition) or MBM with the corresponding lithium (Li^+ condition) or choline (choline condition) salts replacing sodium chloride and sodium bicarbonate. The data are expressed as $pmol \cdot oocyte^{-1} \cdot h^{-1}$ and are the averages ± S.D. of at least 10 oocytes. Abbreviations: Ala, alanine; Gly, glycine; Gln, glutamine; Ser, serine; Thr, threonine; CysH, cysteine; Arg, arginine; His, histidine, Leu, leucine; Phe, phenylalanine; Trp, tryptophan; AIB, 2-aminoisobutyric acid.

Experiments involving the Na^+-dependent transport of 200 μM MeAIB (Fig. 2-1) and AIB (data not shown) by isolated oocytes showed a slow uptake that was linear for at least 2 hours. Although 90% of 50 μM [^{3}H]AIB uptake was prevented by the addition of 5 mM unlabelled AIB, MeAIB at the same inhibitor concentration inhibited only 26% of the Na^+-dependent AIB transport (Table 2-2). Similar results were obtained when the Na^+-dependent transport of characteristic System ASC substrates such as alanine, serine, threonine, and cysteine was challenged with excess MeAIB (Table 2-2). To test for MeAIB-dependent trans-inhibition, another defining property of System A (Handlogten et al., 1981; Kilberg et al., 1985), the intracellular concentration of MeAIB was brought to 1.3 mM by incubating oocytes overnight in MBM containing 10 mM MeAIB. There was no detectable decrease in Na^+-dependent AIB transport by the MeAIB-loaded cells (data not shown). Although it is possible that a higher intra-oocyte MeAIB concentration might have caused trans-inhibition of AIB uptake, this is unlikely given the inability of excess MeAIB to cis-inhibit AIB transport (Table 2-2). Two other systems could possibly account for the Na^+-dependent AIB uptake by the oocyte, System $B^{o,+}$ or System ASC. These two activities can be easily distinguished because of the acceptance of cationic amino acids by the $B^{o,+}$ carrier (Van Winkle et al., 1985). The Na^+-dependent uptake of AIB was measured in the presence of increasing concentrations of

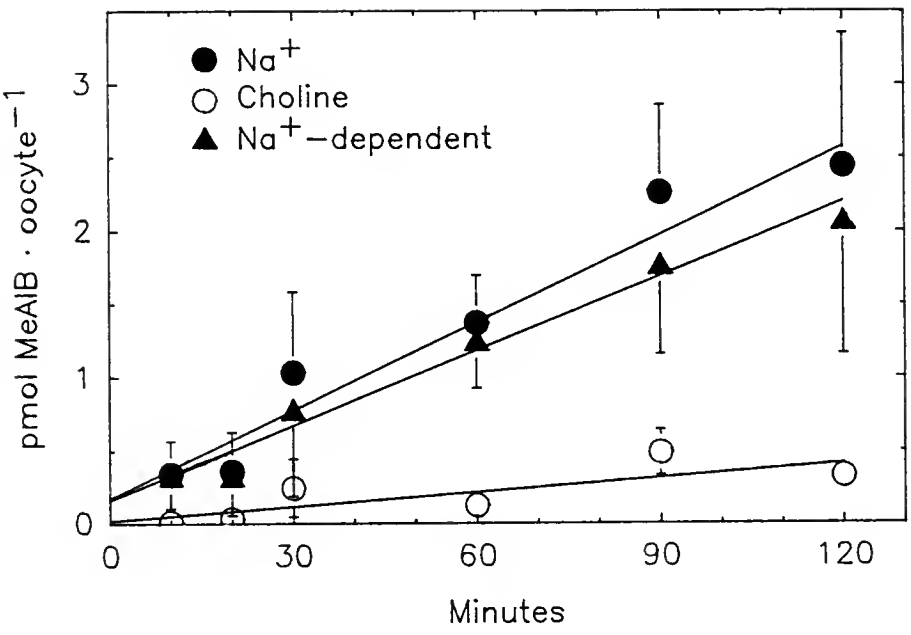

Figure 2-1. Time-course for transport of MeAIB into X. laevis oocytes. The Na^+-dependent and Na^+-independent transport of 200 μM [^{14}C]MeAIB was measured at 20°C as described in the Materials and Methods section. The data are expressed as pmol·oocyte^{-1} and each point represents the average ± S.D. of at least 8 individual oocytes. Where not shown, the standard deviation bars are contained within the symbol.

Table 2-2. MeAIB inhibition of Na^+-dependent transport for selected amino acids.

Substrate	MeAIB	Velocity	Percent of Control
AIB	-	4.9 ± 1.2	
	+	3.6 ± 1.1	74
Ala	-	20.9 ± 3.6	
	+	28.7 ± 4.8	137
Ser	-	23.0 ± 5.4	
	+	20.1 ± 1.7	87
Thr	-	30.6 ± 4.7	
	+	24.5 ± 5.1	80
CysH	-	43.8 ± 9.3	
	+	54.0 ± 10.3	123

The Na^+-dependent transport of 50 μM [^{3}H]AIB, [^{3}H]alanine, [^{3}H]serine, or [^{3}H]threonine was measured for 60 minutes at 20°C in the presence or absence of 5 mM MeAIB as described in the Materials and Methods section. The [^{3}H]cysteine was handled in an equivalent manner except that the concentration was 100 μM and 1 mM dithiothreitol was included in the uptake mixture. The Na^+-dependent uptake rates are reported as pmol·oocyte^{-1}·h^{-1} and are the averages ± S.D. of at least 10 individual oocytes. The addition of unlabelled AIB (5 mM) decreased Na^+-dependent [^{3}H]AIB uptake from 4.9 ± 1.2 to 0.45 ± 0.2 pmol·oocyte^{-1}·h^{-1}, a 91% reduction.

arginine with or without serine (Fig. 2-2). Arginine concentrations up to 10 mM did not significantly affect Na^+-dependent AIB uptake, but the addition of as little as 1 mM serine, a System ASC substrate, nearly eliminated all alkali ion dependent AIB uptake. Furthermore, the Na^+-dependent transport of 50 μM AIB (4.9 ± 1.2 pmol•oocyte^{-1}•h^{-1}) was decreased to 0.5 ± 0.2, 0.4 ± 0.2, and 0.9 ± 0.4 pmol•oocyte^{-1}•h^{-1} by the presence of 5 mM threonine, leucine, or histidine, respectively. These results are indicative of a carrier with broad substrate specificity, such as System ASC (Kilberg et al., 1981; Christensen, 1984). Interestingly, the concentration-dependent decrease of Na^+-independent AIB transport by arginine suggests the presence of the recently defined System $b^{o,+}$ (Van Winkle et al., 1988), a Na^+-independent transport system with the ability to accept neutral as well as cationic amino acid substrates. The saturability of Na^+-independent AIB uptake was verified by the strong inhibitory activity of 5 mM unlabelled AIB on the Na^+-independent transport of 50 μM [^{3}H]AIB. In this experiment, only 15% of the total [^{3}H]AIB entry into oocytes in the absence of sodium could be attributed to non-saturable uptake (i.e., non-carrier mediated).

In contrast to several mammalian cell types (White, 1985), the transport of positively-charged (basic) amino acids into oocytes was found to occur by Na^+-dependent as well as Na^+-independent processes. When measured in the

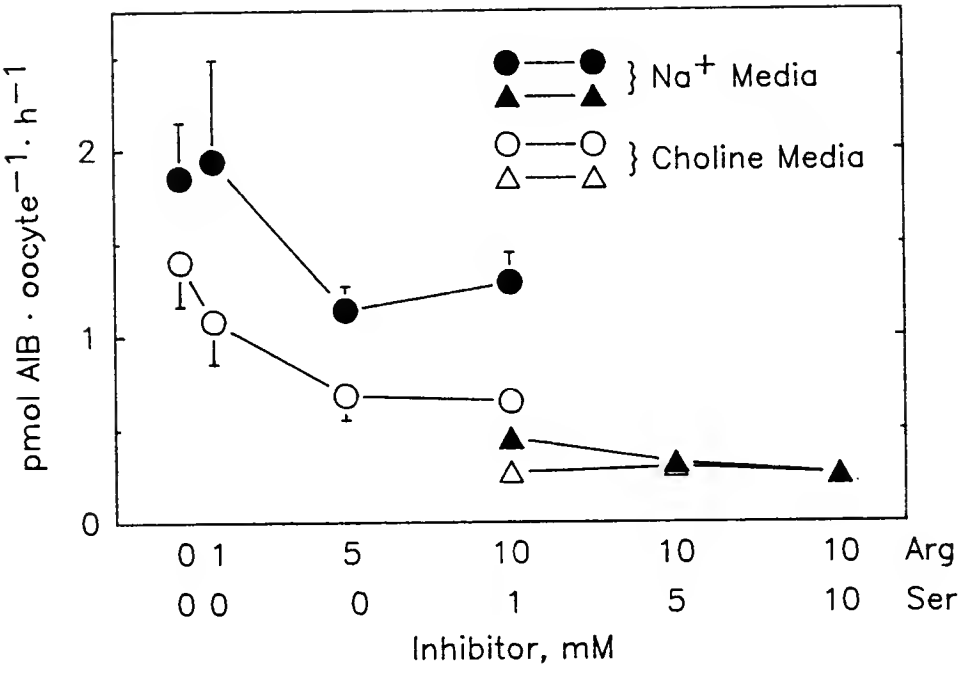

Figure 2-2. Arginine and serine inhibition of AIB transport. The transport of 50 μM [^{3}H]AIB by oocytes was measured in Na^+-containing (filled symbols) or Na^+-free (open symbols) media for 60 minutes at 20°C as described in the Materials and Methods section. Inhibitory amino acids (circles = arginine, triangles = serine + arginine) were included in the uptake mixtures at 1, 5, or 10 mM. Choline chloride was used to maintain the osmolarity of all uptake mixtures at a constant level. Data are expressed as pmol·oocyte^{-1}·h^{-1} and each point represents the average ± S.D. of at least 10 oocytes. Where not shown, the S.D. bars are contained within the symbol.

presence of other amino acids, Na^+-dependent arginine transport was inhibited by the presence of not only other cationic amino acids such as lysine, homoarginine, ornithine, and 2-amino-3-guanidinopropionic acid (AGPA), but by zwitterionic amino acids such as alanine, threonine, and leucine as well (Fig. 2-3). Cysteic acid, which is negatively-charged at physiological pH, had virtually no effect on Na^+-dependent [^{3}H]arginine transport. Consistent with the lack of arginine inhibition of Na^+-dependent AIB uptake (Fig. 2-2), AIB was a relatively poor inhibitor of Na^+-dependent arginine uptake. These results, which suggest the existence in oocytes of a transport system capable of interacting with both neutral and positively-charged amino acids, are similar to those reported by Van Winkle et al. (1985) for a Na^+-dependent carrier identified in pre-implantation mouse blastocysts. The murine system, which was named System $B^{o,+}$, displayed mutual competition among alanine, lysine, and 3-aminoendobicyclo-(3,2,1)-octane-3-carboxylic acid (BCO). The lack of inhibition by cysteic acid is in agreement with the observation that Na^+-dependent alanine transport in the blastocyst was insensitive to the presence of cysteine sulfinate, a negatively-charged amino acid (Van Winkle et al., 1985).

To delineate further the System $B^{o,+}$ activity, mutual inhibition of threonine and arginine was assayed. The transport of Na^+-dependent arginine was shown to be inhibited

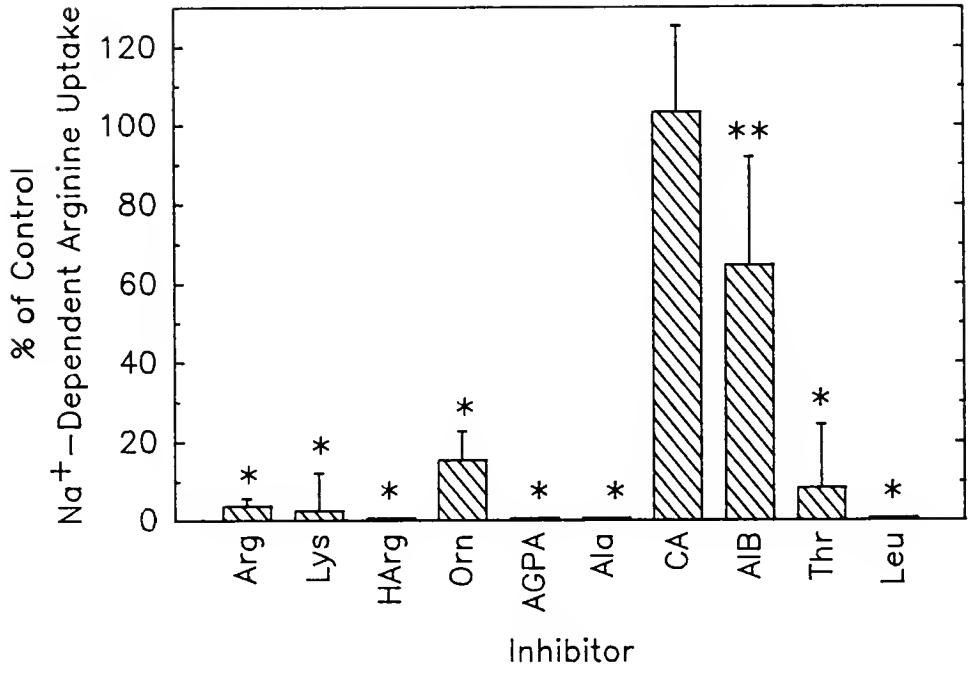

Figure 2-3. Inhibition of Na^+-dependent arginine transport by basic, acidic, and neutral amino acids. The Na^+-dependent transport of 50 μM [^{3}H]arginine in oocytes was assayed for 60 minutes at 20°C as described in the Materials and Methods section. The concentration of the indicated inhibitor was 5 mM in all cases. The data were compiled from two separate experiments and are expressed as the percentage of the transport rate in the absence of inhibition (7.7 ± 4.2 and 7.8 ± 2.5 pmol·oocyte^{-1}·h^{-1} for the two experiments). Each bar represents the average Na^+-dependent transport rate for at least 10 oocytes. Both the Na^+-containing and the Na^+-free buffers contained the inhibitory amino acid. The statistical significance is indicated with asterisks (*$p<0.005$, and ** $p<0.025$).

almost entirely by 5 mM threonine (Fig. 2-3). Conversely, when the Na^+-dependent transport of threonine was challenged with excess arginine, only 50 to 60% was inhibited (Fig. 2-4). The remaining Na^+-dependent threonine transport was eliminated by the inclusion of 1-10 mM serine in the uptake mixture. These data demonstrate the presence of at least two Na^+-dependent carriers in the oocyte plasma membrane. One carrier is able to interact with both positively-charged and neutral amino acids (Na^+-dependent arginine transport inhibited by threonine, Table 2-3), analogous to System $B^{o,+}$, and the other with zwitterionic amino acids only (serine-sensitive portion of Na^+-dependent threonine transport, Fig. 2-4). The substrate specificity and the lack of interaction with MeAIB (Table 2-2) of the latter system argues for analogy to System ASC.

The Na^+-independent transport of positively-charged amino acids by a number of eukaryotic cell types has been shown to be mediated by a process termed System y^+ that is relatively pH insensitive and stereo-selective (White, 1985). Data obtained with a broad spectrum of cell types suggest that System y^+ is distinct from those routes characteristically utilized by neutral amino acids, although it has been demonstrated that a neutral amino acid and a Na^+ can competitively inhibit the carrier (Christensen and Handlogten, 1969; White, 1985). The Na^+-independent transport of arginine by oocytes was inhibited by positively

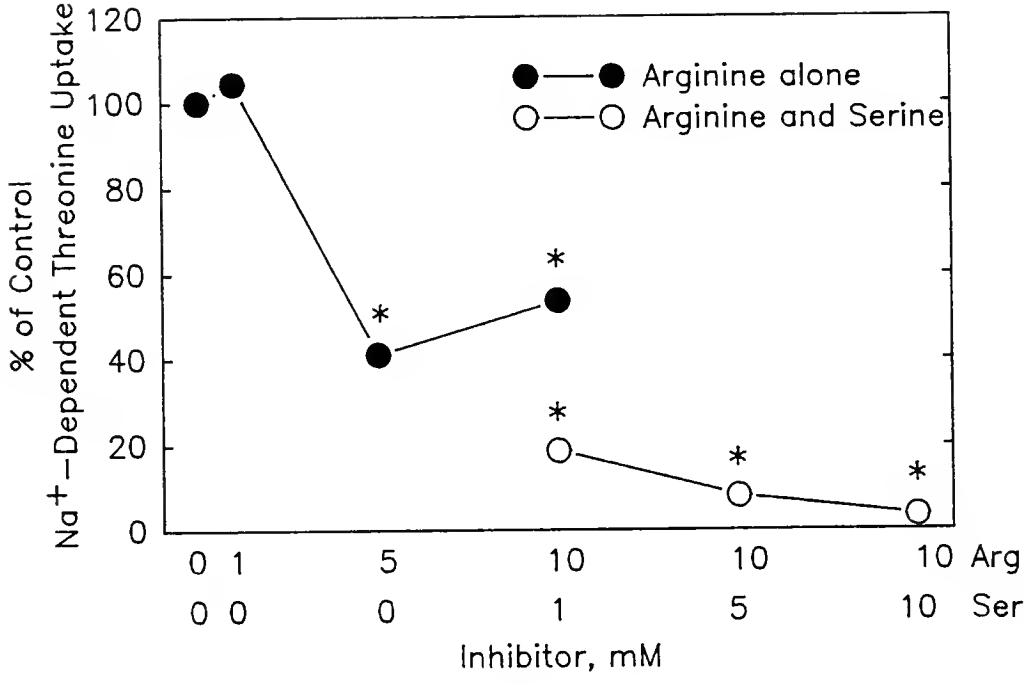

Figure 2-4. Arginine and serine inhibition of Na^+-dependent threonine transport. The Na^+-dependent transport of 50 μM [^{3}H]threonine by oocytes was measured for 60 minutes at 20°C as described in the Materials and Methods section. As indicated, inhibitory amino acids were included in the uptake mixtures at 1, 5, or 10 mM. Choline chloride was used to maintain the osmolarity of all uptake mixtures at a constant level. The data are expressed as the percentage of the Na^+-dependent transport rate in the absence of inhibition. The uninhibited threonine transport rates in Na^+-containing and Na^+-free buffers were 48.3 ± 9.2 and 28.6 ± 5.3 pmol·oocyte^{-1}·h^{-1}, respectively. An asterisk indicates that the value is statistically ($p<0.05$) different from control.

Table 2-3. Inhibition analysis of Na^+-independent leucine transport.

Inhibitor	Velocity	Percent of Saturable
none	19.9 ± 6.6	100
Leu	0	0
Phe	0	0
His	2.7	14
Thr	4.3	22
BCH	8.5	43

The Na^+-independent transport of 50 μM [^{3}H]leucine in oocytes was measured in the presence or absence of 5 mM competitor amino acid as described in the Materials and Methods section. Transport was carried out for 60 minutes at 20°C. The saturable leucine transport rate was calculated by subtracting the rate observed in the presence of excess unlabelled test amino acid from that observed in the absence of inhibitor. The data are expressed as $pmol \cdot oocyte^{-1} \cdot h^{-1}$ or as the percent of the saturable transport rate in the absence of inhibition. The results represent the average of at least 10 oocytes.

charged amino acids, but neutral and negatively-charged amino acids were significantly less effective (Fig. 2-5). Over 80% of the saturable Na^+-independent transport of 50 μM arginine could be eliminated by 5 mM lysine, homoarginine, or ornithine. System y^+ exhibits increasing affinity for substrate molecules as the length of the side-chain increases. For example, the K_i values for inhibition of arginine uptake in HTC cells (White and Christensen, 1982) and human fibroblasts (White et al., 1982) are the lowest for homo-arginine, with four methylene groups in the side-chain, and the highest for 2-amino-3-guanidinopropionic acid (AGPA), for which the side-chain contains only one methylene group. Consistent with the data obtained for fibroblasts (White et al., 1982), 5 mM AGPA decreased arginine uptake by only 20% or less. Essentially equivalent results were obtained with lysine as the test substrate (data not shown).

In an effort to investigate the effect of charge alone on an amino acid's ability to inhibit System y^+, arginine transport was challenged with an excess of histidine at two different pH values (Fig. 2-6). At pH 6.0, approximately half of the histidine molecules possess a net positive charge and half are zwitterionic in form. At pH 8.0, most of the histidine molecules are zwitterionic. Thus, it is possible to separate the inhibitory effect of charge from that imparted by the size, shape, and hydrophilicity of the amino acid's side-chain. The data of Figure 2-6 illustrate that

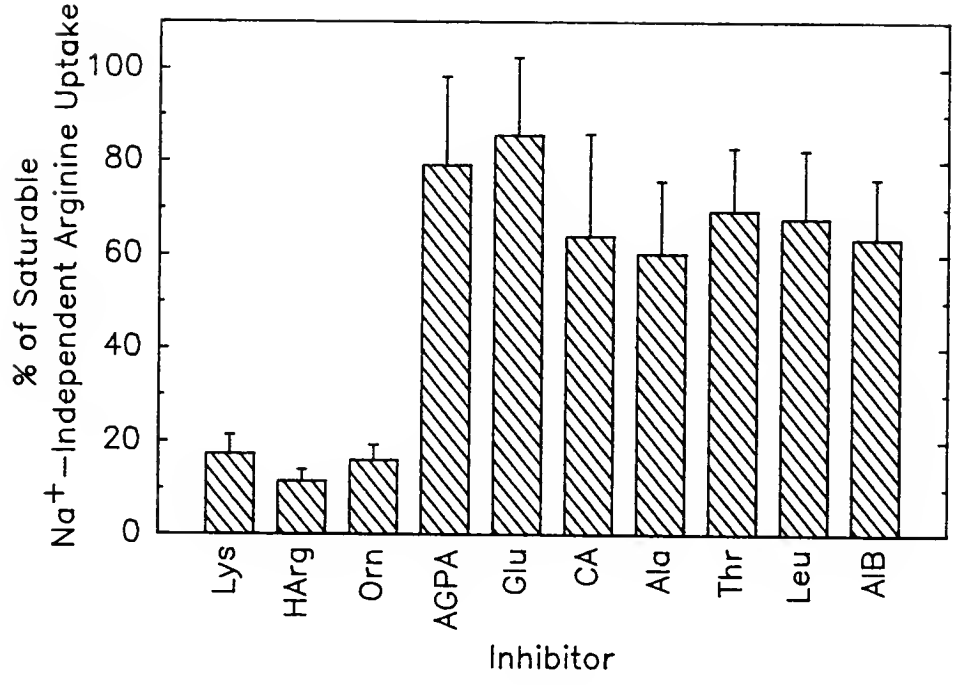

Figure 2-5. Inhibition of saturable Na^+-independent arginine transport by basic, acidic, and neutral amino acids. The Na^+-independent transport by oocytes of 50 μM [^{3}H]arginine was measured for 60 minutes at 20°C as described in the Materials and Methods section. The concentration of the indicated inhibitor was 5 mM in all cases. The data are expressed as the percentage of the saturable transport rate in the absence of inhibition (13.1 ± 1.5 pmol·oocyte^{-1}·h^{-1}). The total saturable transport rate was calculated by subtracting the uptake rates measured in the presence or absence of 5 mM unlabelled arginine. Each bar represents the average transport rate ± S.D. of at least 10 individual oocytes.

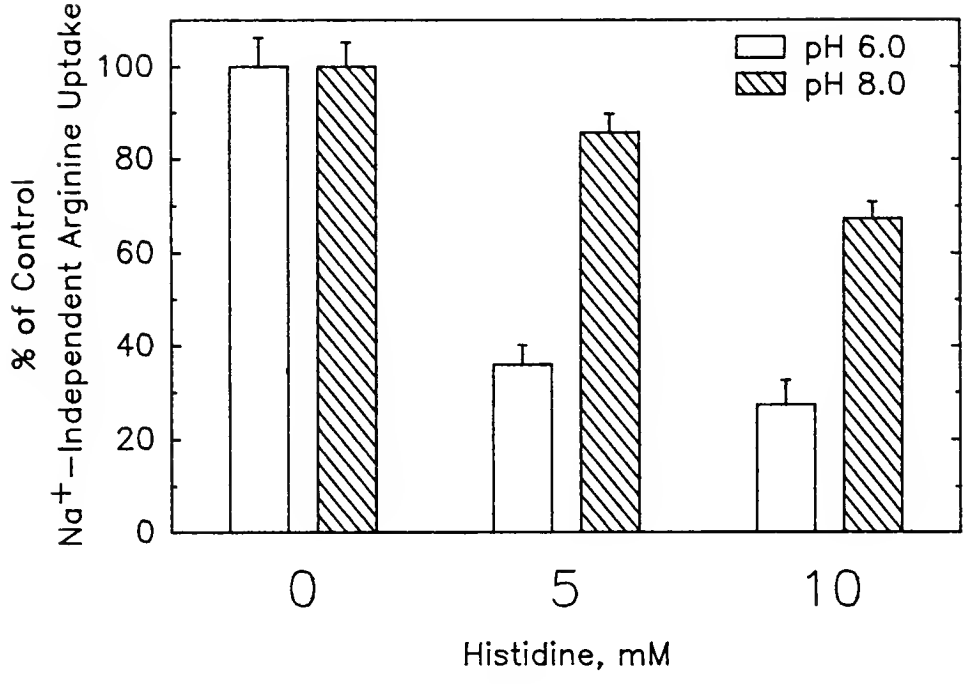

Figure 2-6. The pH dependence for histidine inhibition of Na^+-independent arginine transport. The Na^+-independent transport by oocytes of 50 μM [^{3}H]arginine was measured for 60 minutes at 20°C in the presence or absence of 5 mM unlabelled histidine at pH 6.0 or 8.0. The data are expressed as the percentage of the transport rate in the absence of inhibition measured at the same pH (pH 6 = 30.4 ± 1.9, pH 8 = 22.3 ± 1.2 pmol·oocyte^{-1}·h^{-1}) and represent the averages ± S.D. of at least 10 separate cells.

the charged form of histidine was a more effective inhibitor of Na^+-independent arginine transport than the neutral form. At pH 6.0, 10 mM histidine inhibited approximately 70% of arginine transport, while at pH 8.0, only 30% was blocked. As seen for System y^+ in other cell types (White et al., 1982; White and Christensen, 1982), Na^+-independent arginine transport was found to be insensitive to inhibition by H^+ over a wide range, the rate at pH 6.0 being equal to, or slightly higher than, that at pH 8.5 (Fig. 2-6). In agreement with previous results regarding the stereo-selectivity of Na^+-independent arginine transport (White et al., 1982; White and Christensen, 1982), L-arginine uptake (50 μM) was inhibited approximately 95% by 5 mM unlabelled L-arginine (20.8 ± 2.9 versus 0.98 ± 0.2 $pmol \cdot oocyte^{-1}, \cdot hr^{-1}$), whereas the same concentration of D-arginine was much less effective, decreasing arginine transport by about 45% (20.8 ± 2.9 versus 11.7 ± 2.5 $pmol \cdot oocyte^{-1} \cdot hr^{-1}$).

In a variety of eukaryotic cells, the transport of amino acids possessing aromatic or branched side-chains is mediated largely by the System L amino acid carrier (Shotwell et al., 1983). System L is characterized by Na^+-independence, pH and N-ethylmaleimide (NEM) insensitivity, trans-stimulation, and affinity for the non-metabolizable analog, 2-aminobicyclo-[2,2,1]-heptane-2-carboxylic acid (BCH) (Vadgama and Christensen, 1985a,b). In erythrocytes, there exists a Na^+-independent neutral amino acid carrier

distinct from System L (Vadgama and Christensen, 1985a,b; Fincham et al., 1985). This carrier has been named System asc; the letters chosen to reflect the substrate similarity to the Na^+-dependent System ASC (i.e. alanine, serine, cysteine, and threonine) and the use of lower case to indicate Na^+-independence (Bannai et al., 1984). Although the subset of amino acids characteristically transported by Systems L and asc do overlap, the two carriers can be functionally distinguished (Vadgama and Christensen, 1985a,b). Preliminary evidence in oocytes indicated the presence of multiple Na^+-independent neutral amino acid carriers. As a result, the transport of threonine, BCH, and leucine was monitored to determine whether these activities appeared similar to those already described (i,e, Systems L and asc). Historically, the Na^+-independent uptake of leucine and BCH has been considered relatively specific for System L, and recent evidence suggests that threonine is a good test substrate for System asc (Vadgama and Christensen, 1985a,b).

When Na^+-independent leucine transport was assayed, phenylalanine, histidine, and threonine eliminated greater than 70% of the saturable leucine uptake (Table 2-3). The branched-chain amino acid analog BCH was a relatively poor inhibitor when compared to leucine itself, lowering the transport of leucine by about 60%. To test for trans-stimulation, oocytes were incubated for approximately 20

hours in MBM containing 10 mM unlabelled leucine. This pre-loading with leucine resulted in a greater than 2-fold stimulation of saturable Na^+-independent leucine transport (12.0 ± 0.9 versus 28.2 ± 4.8 pmol·oocyte^{-1}·h^{-1}, $p<0.01$). An analogous experiment utilizing oocytes preloaded with alanine, a presumed substrate for System asc, if present, showed no change in saturable Na^+-independent alanine transport.

The Na^+-independent uptake of BCH was inhibited most effectively by an excess of phenylalanine, leucine, or BCH itself, but was resistant to inhibition by threonine, alanine, or serine (Fig. 2-7). AIB, cysteic acid, and arginine were also totally ineffective as inhibitors (data not shown). As can be seen in Figure 2-7, a portion of the saturable Na^+-independent threonine transport in oocytes can be inhibited by leucine and BCH even though, as noted above, threonine was a relatively poor inhibitor of BCH transport. In two separate experiments, 10 mM threonine was able to inhibit only 31% (not shown) and 35% (Fig. 2-7) of saturable Na^+-independent BCH transport. This is in agreement with the inability of threonine, probably the inhibitor/substrate of choice to monitor System asc, to inhibit BCH uptake via System L in human erythrocytes (Vadgama and Christensen, 1985b).

Differences in stereo-selectivity between threonine and BCH transport into oocytes were also apparent (Fig. 2-7).

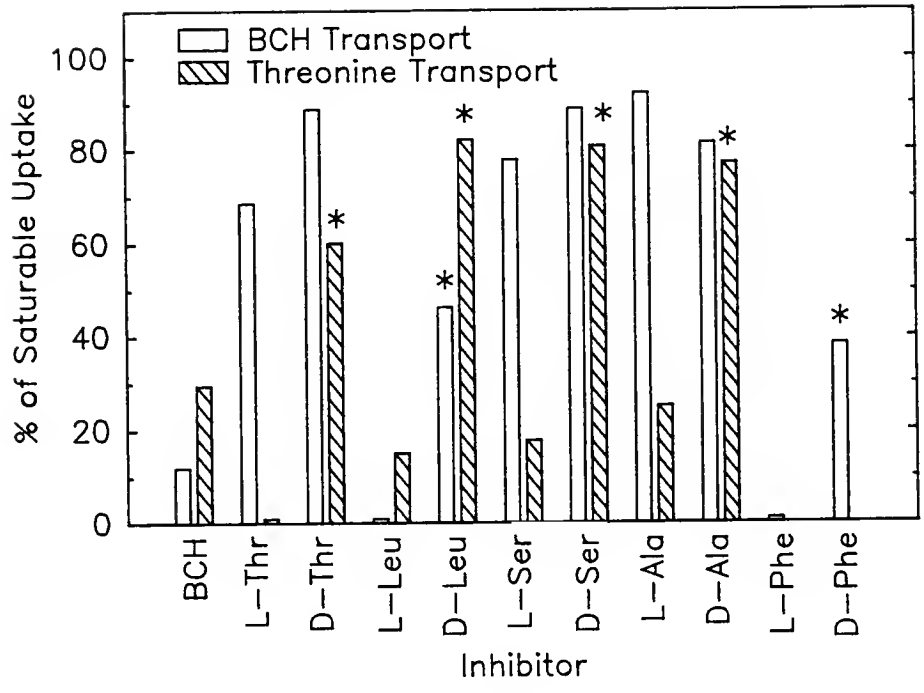

Figure 2-7. Stereo-selectivity for inhibitors of Na^+-independent BCH or threonine transport. The Na^+-independent transport of 200 μM [^{3}H]BCH or [^{3}H]threonine by oocytes was measured for 60 minutes at 20°C in the presence or absence of the indicated inhibitory amino acid at a concentration of 10 mM. Each bar represents the average of at least 10 oocytes and the standard deviations, omitted for clarity, were typically 20% or less. An asterisk indicates that the difference in effectiveness between the D- and L-isomers is statistically significant ($p<0.005$).

Na^+-independent threonine transport was relatively stereospecific, approximately 75-80% of its saturable transport was inhibited by the L-forms of alanine, serine, and leucine. However, the D-forms of these amino acids inhibited only 20% or less of threonine uptake. In contrast, the agency responsible for the Na^+-independent, saturable transport of BCH is less stereo-specific, as both D-leucine and D-phenylalanine inhibited greater than 50% of the saturable uptake (Fig. 2-7).

Discussion

The data shown in Table 2-1 demonstrate that amino acid uptake in Xenopus laevis oocytes occurs by Na^+-dependent as well as Na^+-independent means. Greater than 50% of the total transport of 6 of the substrates tested (glycine, threonine, AIB, glutamine, leucine, and cysteine) was shown to be dependent upon the presence of sodium. Conversely serine, arginine, histidine, phenylalanine, and tryptophan were transported primarily by Na^+-independent means. Although variations in transport rates for different batches of oocytes were often observed for individual amino acids, leucine transport was found to be particularly variable. While in most experiments the great majority of leucine transport was Na^+-independent, occasionally a sizeable portion was Na^+-dependent. For alanine, transport was mediated equally by Na^+-dependent and independent processes. Such would be the case if transport systems analogous to

Systems ASC and asc, to be described more fully below, exist in the oocyte plasma membrane.

The low rates of Na^+-dependent MeAIB or AIB transport in oocytes make their use for routine assays of endogenous System A, if present at all, difficult, but this low activity should prove helpful in experiments designed to detect elevated levels of carrier following micro-injection of foreign mRNA coding for that particular transport protein. Although Na^+-dependent uptake of the System A-specific substrate MeAIB was detected, when tested for either trans- or cis-inhibition, Na^+-dependent uptake of AIB was insensitive to the presence of intra- or extra-cellular MeAIB. In freshly isolated hepatocytes in suspension, the Na^+-dependent entry of MeAIB is almost totally restricted to System A, whereas that of AIB is slightly less restricted, approximately 10% of its transport is the result of System ASC mediation (Kilberg et al., 1981). AIB entry into CHO-K1 cells has been shown to be mediated by a combination of Na^+-dependent and Na^+-independent agencies (Bass et al., 1981; Shotwell et al., 1981; Moffett et al., 1983). The Na^+-dependent portion has been shown to be due to System A (60%) and System ASC (10%), whereas System L (30%) has been shown to be responsible for the majority of the Na^+-independent entry. Similarly, in oocytes the transport of AIB appears to be the result of a combination of multiple distinct transport activities. Approximately 75% of the Na^+-independent

transport of AIB in oocytes can be eliminated by arginine, suggesting an activity analogous to System $b^{o,+}$ (Van Winkle et al., 1988), and the remaining 25% by the addition of an excess of serine (Fig. 2-2). We find that threonine and serine, while mutually inhibitory, are not effective inhibitors of the System L substrate BCH (e.g., Fig. 2-7). Thus, the serine-inhibitable Na^+-independent AIB transport may be indicative of System asc activity (Vadgama and Christensen, 1985a,b). System $b^{o,+}$ is a Na^+-independent transporter that accepts both neutral and cationic amino acids in a manner similar to the Na^+-dependent System $B^{o,+}$. System $b^{o,+}$ has a more limited substrate specificity than System $B^{o,+}$ and has been shown to exhibit decreased reactivity with amino acids having large, branched side-chains such as BCH (Van Winkle et al., 1988). The Na^+-dependent portion of AIB transport is insensitive to inhibition by arginine, but easily inhibitable by an excess of serine. Therefore, it appears that the majority of Na^+-dependent AIB transport in oocytes is mediated by a transporter with characteristics similar to System ASC (Van Winkle et al., 1988).

With regard to Na^+-dependent transport of naturally-occurring neutral amino acids, the evidence presented points to mediation by activities similar to Systems ASC and $B^{o,+}$. The assignment of Na^+-dependent alanine transport in oocytes to System A by Jung et al. (1984) was based on pH sensitivity and stereo-selectivity, even though alanine uptake was not

inhibited by the System A-specific probe, MeAIB. Furthermore, the precise pH at which alanine transport declined cannot be gauged as the actual data were not published. We believe that affinity for amino acids with N-mono-methylation such as MeAIB, to be a hallmark of System A. Using this criterion, the results presented indicate that the Na^+-dependent uptake of many neutral amino acids by Xenopus oocytes is mediated by MeAIB-insensitive carrier(s). Using threonine as an example, we find that only about 50% of its Na^+-dependent uptake is inhibitable by an excess of arginine (System $B^{0,+}$), whereas the remainder is strongly inhibited by serine and is probably mediated by an activity similar in scope and properties to the ASC system.

The Na^+-independent transporters were found to represent the major pathways for accumulation of neutral amino acids by Xenopus oocytes. The processes that appear to be responsible are, in general terms, analogous to Systems L, asc, and $b^{0,+}$ described for other cell types (Vadgama and Christensen, 1985a,b; Van Winkle et al., 1988). The distinguishing feature of System $b^{0,+}$ is the ability to accept cationic as well as neutral amino acids (Van Winkle et al., 1988). In the oocyte, the Na^+-independent uptake of threonine is mediated by processes that appear similar in many respects to Systems $b^{0,+}$ and asc as evidenced by the partial inhibition of its transport by the cationic amino acid arginine (data not shown). Increasing the concentration of unlabelled arginine

from 5 to 10 mM caused no further inhibition of Na^+-independent threonine transport, demonstrating the ability of arginine to distinguish between two distinct activities rather than merely exhibiting partial inhibition of a single process. The remainder of the threonine Na^+-independent transport was saturable by serine. This result is indicative of System asc given the relatively poor inhibitory action of serine and threonine on System L-mediated BCH transport. A carrier analogous to System y^+ appears to be responsible for most of the Na^+-independent arginine transport. The cationic forms of lysine and histidine were effective in blocking arginine uptake. For the latter amino acid, the importance of the net positive charge was demonstrated directly by altering the pH of the assay medium. The previously reported insensitivity to pH changes between 6 and 8 of the carrier itself (White, 1985) was confirmed for the oocyte.

With regard to active accumulation of amino acids by the oocyte; i.e., Na^+-dependent transport, an activity that appears to be analogous to System $B^{0,+}$ may predominate. Our observations support a similar proposal by Van Winkle (1988) in a recent review. However, given the resistance to arginine inhibition of a portion of Na^+-dependent threonine and AIB transport, the presence of a carrier preferring zwitterionic amino acids only, such as System ASC, is also indicated. When expressed on a per milligram non-yolk protein basis, the rates of amino acid transport measured in

oocytes are, on average, considerably slower than that observed in other cell types. For example, cationic amino acids, such as arginine, homoarginine, and lysine, are transported into human fibroblasts at a rate of approximately 1 $nmol \cdot mg^{-1} protein \cdot min^{-1}$ (White *et al.*, 1982). The same amino acids are transported into *Xenopus* oocytes at a rate which is only 3% of the fibroblast rate. Similarly, the neutral amino acids alanine, serine, and threonine are transported into oocytes at rates that are only one-fiftieth of that seen in rat hepatocytes. These slow rates of amino acid transport in oocytes may be a reflection of the relatively quiessent metabolic state characteristic of fully-grown germ cells. In summary, it is clear that amino acids enter defolliculated *Xenopus* oocytes by means of several distinct transport activities which correspond, in general characteristics, to systems observed in other animal cells.

CHAPTER III

EFFECT OF MEIOTIC MATURATION ON THE TRANSPORT OF AIB, THREONINE, AND LEUCINE IN *XENOPUS LAEVIS* OOCYTES

Introduction

Fully-grown, stage 6 (Dumont, 1972) *Xenopus laevis* oocytes, which are arrested in prophase of the first meiotic division, can be induced to mature *in vitro* by the action of specific hormones or growth factors. It is only the fully-matured oocytes, now called eggs, that are fertilizable. *In vivo*, meiotic maturation is triggered by gonadotropins released from the pituitary gland (Wasserman, 1986). The hormones interact with the follicle cells surrounding the oocytes, stimulating them to synthesize and secrete progesterone. The maturation process commences following the interaction of progesterone with the oocytes. During the course of this maturation process, which culminates in a meiotic blockade at metaphase of the second division, the oocyte nuclear envelope breaks down. The disappearance of the nucleus, also called the germinal vesicle, signals the completion of maturation and can be easily verified *in vitro* by the presence of a non-pigmented spot at the brown-colored animal pole. Germinal vesicle breakdown (GVBD) can also be gauged by fixing the oocyte in trichloroacetic acid and confirming the absence of the nucleus following dissection.

In this report, the terms "GVBD" and "maturation" are used interchangeably.

The most widely studied hormones used to induce maturation *in vitro* are progesterone and insulin. Much work has been carried out in recent years in attempts to elucidate the precise mechanism involved in progesterone- or insulin-induced GVBD (Stith and Maller, 1984; Maller, 1987). Although there is still debate with regard to the nature and order of events taking place, there is general acceptance that the induction of GVBD by either of these hormones is accompanied by the following: 1) a decrease in the intra-oocyte concentration of 3',5'-cyclic adenosine monophosphate (cAMP); 2) an increase in overall protein synthesis; 3) an increase in total protein phosphorylation; and 4) the activation of a specific cytoplasmic factor, named the maturation promoting factor (MPF), which is responsible for mediation of the late meiogenic events of the maturation process (Taylor and Smith, 1987; Maller, 1987).

Recognition of the existance of MPF arose from experiments which demonstrated the meiotic maturation of oocytes after they had been injected with a portion of the cytoplasm obtained from a fully-matured (i.e. post-GVBD) egg (Masui and Markert, 1971; Smith and Ecker, 1971). In contrast to the hormone-induced event, which takes from 6 to 12 hours, MPF-induced maturation is relatively rapid, occurring in less than 2 hours. Further, this factor appears

to function post-translationally as its effect is unaltered by the presence of the protein synthesis inhibitor, cycloheximide (Taylor and Smith, 1987). The MPF from Xenopus eggs has been partially purified (Wu and Gerhart, 1980) and shown to possess a protein kinase activity. In agreement with this finding, earlier work (Maller et al., 1977) had demonstrated an increase in total protein phosphorylation following the transfer of mature oocyte cytoplasm into prophase-arrested oocytes. This increase in protein phosphorylation is also observed following incubation of oocytes in medium containing progesterone or insulin. In addition, the action of either of these hormones results in a rapid decline in the cytoplasmic level of cAMP (Stith and Maller, 1985; Maller, 1987); progesterone acting by inhibition of adenylate cyclase and insulin by activation of phosphodiesterase (Maller, 1987). Interestingly, insulin, as well as insulin-like growth factor-1 (IGF-1), has been shown, in vitro, to bring about a decrease in the activity of adenylate cyclase (Sadler and Maller, 1987). Conversely, activators of adenylate cyclase, such as cholera toxin, and inhibitors of phosphodiesterase, such as theophylline and isobutylmethylxanthine, have been shown to inhibit hormone-induced GVBD in oocytes (Stith and Maller, 1984,1985; Sadler and Maller, 1987).

Although the mechanisms by which progesterone and insulin bring about meiotic maturation in oocytes appear to

be very similar, both operating by causing a decrease in the cytoplasmic concentration of cAMP, evidence has demonstrated that the two pathways are actually quite distinct. For example, following treatment with either progesterone or insulin, Xenopus oocytes have been shown to have elevated intracellular pH (pH_i) (Lee and Steinhardt, 1981) as well as increased phosphorylation of ribosomal protein S6 (Hanocq-Quertier and Baltus, 1981). Inclusion of cholera toxin in the incubation medium, while able to block both progesterone- and insulin-induced GVBD, inhibits only the progesterone-mediated increase in pH_i and S6 phosphorylation while having no effect on the increases caused by insulin (Stith and Maller, 1984). In addition, incubation of oocytes in Na^+-free medium containing amiloride, a Na^+/H^+ exchange blocker, prevents the decline in pH_i following treatment with either hormone. However, in the presence of progesterone, the percentage of oocytes undergoing meiotic maturation is unchanged from that in amiloride-free Na^+-containing medium while it is reduced by 60% when insulin is used (Stith and Maller, 1985). This result also indicates that the decrease in pH_i accompanying GVBD is not necessary for maturation to occur.

In addition to insulin's effects on cAMP levels via phosphodiesterase activation, the tyrosine kinase domain of the oocyte insulin receptor has been shown to be intimately involved in catalyzing GVBD. In 1986, Morgan et al. showed

that insulin- (but not progesterone-) induced GVBD could be prevented by the injection of antibodies directed against the kinase domain of the human insulin receptor. Furthermore, injection of antibodies directed against a highly conserved region of the ras gene product (p21) was shown to block insulin-mediated GVBD while having no effect on progesterone-mediated GVBD (Korn et al., 1987). The ras protein is a membrane-bound, guanine nucleotide binding protein that is believed to function in the response of cells to insulin (Kamata and Feramisco, 1984; Heyworth et al., 1985). Among the actions attributed to both insulin (Saltiel et al., 1986) and p21 (Fleischman et al., 1986) is activation of a specific phospholipase C. Activation of phospholipase C results in a transient increase in the intracellular concentrations of diacylglycerol and inositol phosphates as a result of the enzyme's action upon phosphatidylinositol 4,5-bisphosphate (Houslay et al., 1987). The inositol phosphates are involved in the release of Ca^{++} from non-mitochondrial stores (Berridge, 1983). Evidence has also been reported which implies a role for inositol 1,3,4,5-tetrakisphosphate in increasing the permeability of sea urchin egg plasma membrane to Ca^{++} (Irvine and Moor, 1986). Diacylglycerol, on the other hand, interacts with protein kinase C, causing its activation. The tumor promoting agent, TPA (12-O-tetradecanoylphorbol-13-acetate), contains a diacylglycerol-like moiety and has been shown to bring about

the activation of protein kinase C (Nishizuka, 1984), presumably by mimicking the action of diacylglycerol itself. TPA has also been shown to be an effective inducer of GVBD in Xenopus oocytes (Stith and Maller, 1987).

In addition to the changes mentioned above, meiotic maturation of Xenopus laevis oocytes also results in a generalized decline in plasma membrane transport activity to near zero levels (Richter et al., 1984). Following induction of GVBD via progesterone, Richter et al., (1984) noted a dramatic decrease in the uptake of L-alanine, thymidine, chloride, phosphate, and alkali ions. Likewise, the Na^+-dependent transport of L-leucine was shown in earlier work to decrease markedly following progesterone-induced GVBD (Belle' et al., 1976). The protein synthesis inhibitor, cycloheximide, was shown to prevent not only GVBD but the associated decrease in Na^+-dependent leucine transport as well. Because the matured oocytes are normally ovulated into pond water, it has been hypothesized that this decrease in transport activity may serve to minimize the loss of intracellular solute molecules, such as amino acids, through carrier-mediated efflux (Van Winkle, 1988).

This chapter describes an investigation into the effects of progesterone- or TPA-induced GVBD on the transport of leucine, threonine, and AIB. Studies described in Chapter II involving cis-inhibition and trans-stimulation of leucine transport indicate that this amino acid enters oocytes by

means of a carrier similar in many respects to System L (Shotwell et al., 1983). For this reason the saturable, Na^+-independent transport of leucine subsequent to in vitro induced meiotic maturation was investigated. Additional experiments described in Chapter II (Figs. 2-4 and 2-7) indicate that threonine enters oocytes both by Na^+-dependent and Na^+-independent means. Data from this investigation implies that threonine transport is mediated by two previously studied Na^+-dependent transporters: Systems ASC (Kilberg et al., 1981) and $B^{0,+}$ (Van Winkle et al., 1988) and one Na^+-independent transporter, System asc (Vadgama and Christensen, 1985a,b). The effect of GVBD on the relative activities of these carriers was examined by monitoring the Na^+-dependent (System ASC and $B^{0,+}$) and saturable, Na^+-independent (System asc) transport of threonine. Maturation-induced effects on the Na^+-dependent oocyte transport of AIB were also conducted. Although the Na^+-dependent transport of AIB in rat hepatocytes is mediated almost entirely (greater than 90%) by System A, it appears to be transported primarily by a System ASC-like carrier in Xenopus oocytes (see Chapter I).

Materials and Methods

Isolation of Oocytes and Amino Acid Transport

Methodology for the isolation of oocytes and amino acid transport is included in the Materials and Methods section in Chapter II.

Induction of Meiotic Maturation in Oocytes

Manually defolliculated oocytes were incubated in plastic Petri dishes at 20°C in MBM containing either progesterone or 12-O-tetradecanoylphorbol-13-acetate (TPA) for the specified period of time. Progesterone and TPA stock solutions were stored in 0.2 ml aliquots at -20°C. The stock solutions were made by dissolving progesterone in 95% (v/v) ethanol at a concentration of 2 mg/ml (6.4 mM) or TPA at a concentration of 1 mg/ml (1.62 mM) in dimethylsulfoxide (DMSO). Control oocytes for progesterone- or TPA-induced GVBD were incubated in MBM containing ethanol or DMSO respectively, at concentrations equivalent to those in the experimental conditions. Bovine serum albumin (BSA), at a concentration of 0.2% (w/v), was included in the TPA-containing medium to minimize nonspecific adsorption of the phorbol ester to the plastic dish. Meiotic maturation (i.e. GVBD) was verified by placing oocytes in 10% (w/v) trichloroacetic acid for approximately 10 minutes at room temperature followed by dissection using Dumont #5 forceps. Absence of the germinal vesicle was taken as evidence of meiotic maturation. For transport experiments, only those oocytes possessing a white spot at the animal pole were used. Fixation and dissection of such oocytes revealed a perfect correlation between the presence of the spot and the absence of the germinal vesicle.

Results

Initial attempts to induce GVBD in stage 5 and 6 (Dumont, 1972) oocytes with progesterone were without success. After 24 hours of incubation in MBM or O-R2 (82.5 mM NaCl, 2.5 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.0 mM Na_2HPO_4, 5.0 mM HEPES, pH 7.8) (Wallace et al., 1973) containing 10 μM progesterone, all oocytes tested were found to have intact germinal vesicles following trichloroacetic acid fixation and dissection. In 1976, Vitto and Wallace investigated the effects of ouabain, an inhibitor of Na^+/K^+ ATPase, on progesterone-induced GVBD in oocytes. Their results demonstrated that ouabain, while unable to induce GVBD on its own, was able to facilitate maturation when included in the incubation medium in conjunction with progesterone. Further, it was also shown that the omission of K^+ from the incubation buffer had the same effect as the inclusion of ouabain (Vitto and Wallace, 1976). These studies were expanded by Wasserman et al. (1986) with the demonstration of the induction of GVBD in stage 4 oocytes that had been incubated in progesterone-containing, K^+-free O-R2 medium. This same buffer containing 2.5 mM K^+ and progesterone brought about GVBD in stage 5 and 6 oocytes only (Wasserman et al., 1986).

The oocyte incubation medium used in our laboratory, MBM, contains 1 mM K^+. However, in order to avoid interference in determinations of Na^+-dependent amino acid

transport rates, the pH of the MBM was routinely adjusted with KOH, resulting in a final K^+ concentration of 10 to 20 mM. In order to ascertain the effect of this unnaturally high K^+ level on the induction of GVBD via progesterone action, buffers of varying K^+ concentration were tested (Fig. 3-1). In this experiment, 100, 87, and 32% of the oocytes incubated for 12 hours in progesterone-containing MBM with no K^+, 1 mM K^+, or 15 mM K^+, respectively, underwent GVBD. Interestingly, approximately 1 in 10 oocytes incubated in K^+-free MBM without progesterone underwent GVBD as well. Based on this data, all subsequent experiments described in this chapter were carried out using MBM containing a final potassium concentration of 1 mM.

The inclusion of the tumor promoting compound, TPA, in oocyte incubation medium results in the induction of GVBD in stage 5 and 6 oocytes (Stith and Maller, 1987). The concentration of the phorbol ester required to bring about maturation in 50% of the oocytes tested was found to be 150 nM. In agreement with the data of Stith and Maller (1987), TPA at a concentration of 600 nM, in MBM containing 1 mM K^+, was indeed found to be an effective inducer of GVBD (Fig. 3-2). Although 5% of the oocytes tested were shown to have undergone GVBD at 2 and 4 hours, a consistent increase in those maturing was not seen until 6 hours after the addition of TPA. After 10 hours, 90% of the oocytes had undergone GVBD as evidenced by the absence of a germinal vesicle

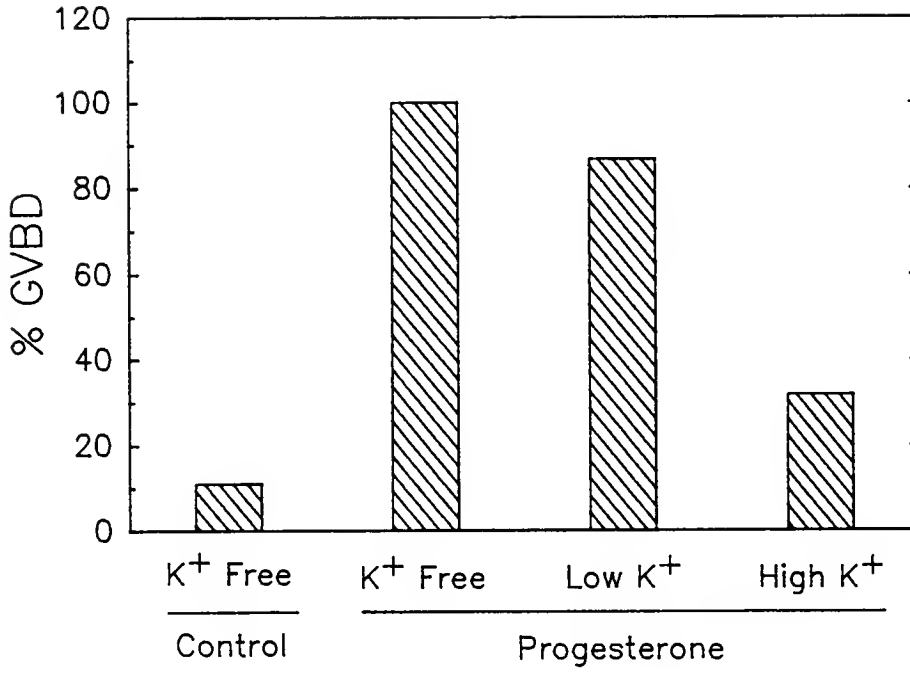

Figure 3-1. Effect of K^+ concentration on progesterone-induced GVBD in oocytes. Oocytes were incubated in K^+-free MBM (K^+ Free Control) or in the following solutions containing 10 μM progesterone: K^+-free MBM (K^+ Free); MBM containing 1 mM K^+ (Low K^+); or MBM containing 15 mM K^+ (High K^+). After 12 hours of incubation at 20°C, the oocytes were fixed in 10% (w/v) trichloroacetic acid and dissected as described in the Materials and Methods section. Absence of the germinal vesicle was taken as evidence of GVBD. The results are expressed as the percentage of oocytes undergoing GVBD.

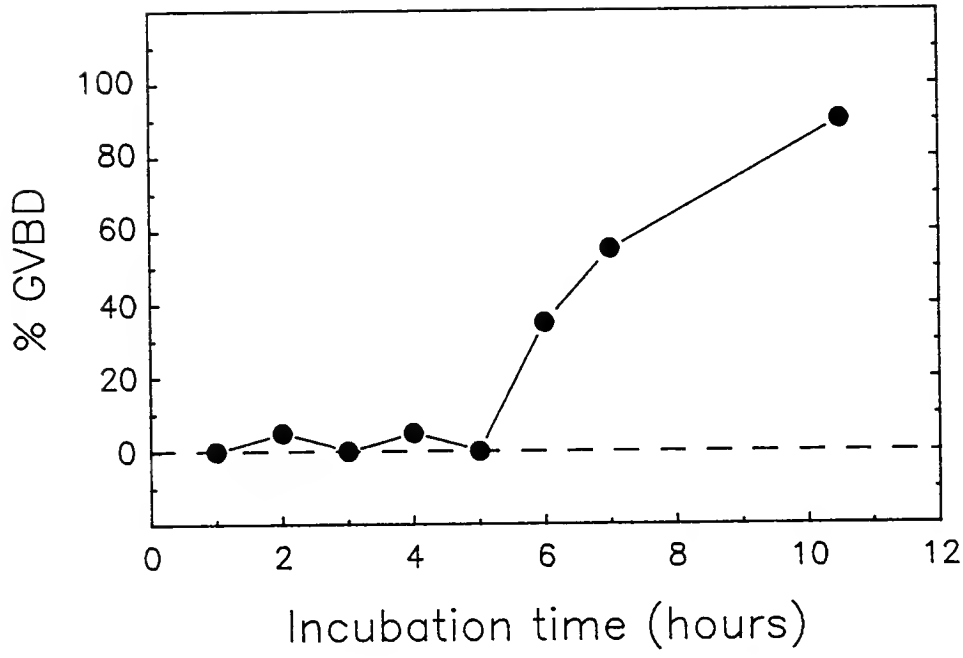

Figure 3-2. Time course of TPA-induced GVBD in oocytes. Oocytes were incubated at 20°C in MBM alone; MBM containing 0.2% (w/v) BSA and 0.04% (v/v) DMSO; or MBM containing 0.2% (w/v) BSA, 0.04% (v/v) DMSO, and 0.6 μM TPA. After varying periods of time, 20 to 40 oocytes were fixed in 10% (w/v) trichloroacetic acid and dissected as described in the Materials and Methods section. Absence of the germinal vesicle was taken as evidence of GVBD. The results are expressed as the percentage of oocytes undergoing GVBD. None of the oocytes incubated in the absence of TPA were found to have undergone GVBD.

following trichloroacetic acid fixation and dissection. The time course of progesterone-induced GVBD was essentially the same as that just described for TPA (data not shown).

Following progesterone- or TPA-induced maturation, the Na^+-dependent transport of threonine and AIB was measured in oocytes (Table 3-1). At a substrate concentration of 50 μM, the Na^+-dependent transport rate of threonine declined 93.1 and 99.6% compared to control following progesterone- or TPA-induced GVBD, respectively. As described in Chapter II, Na^+-dependent threonine transport in oocytes appears to be the result of transport activities analogous to Systems $B^{o,+}$ and ASC. Although no attempt was made to distinguish between mediation by one or the other carrier, the almost complete elimination of Na^+-dependent threonine transport following GVBD indicates that both activities declined equally. Although a decline in the Na^+-dependent AIB transport rate was also observed, the difference was found to be non-significant at the $P<0.05$ level. The saturable, Na^+-independent transport rates of 50 μM threonine and leucine were also shown to decline following induction of GVBD (Table 3-2). In the absence of sodium, the saturable rate of threonine entry declined 78.3% following TPA-induced GVBD. However, the decline following progesterone-induced maturation was only 16% of the control value. The decrease in the rate of leucine entry into oocytes seen following progesterone-induced maturation (63.8%) was also of lesser

TABLE 3-1. Measurement of Na^+-dependent amino acid transport in oocytes following induction of GVBD by progesterone or TPA.

Substrate	Velocity Control	GVBD	% Decrease	P<
Progesterone:				
AIB	0.9 ± 0.4	0.3 ± 0.3	69.4	N.S.
Thr	38.6 ± 21.2	2.7 ± 2.7	93.1	0.025
TPA:				
AIB	3.8 ± 1.6	3.0 ± 2.8	21.7	N.S.
Thr	34.2 ± 7.8	0.1 ± 3.2	99.6	0.005

Germinal vesicle breakdown (GVBD) was induced in oocytes by the inclusion of progesterone (3.2 μM) or TPA (0.6 μM) in the surrounding medium and incubating for 10 to 12 hours as described in the Materials and Methods section. The Na^+-dependent transport of 50 μM [^{3}H]AIB or [^{3}H]threonine was then measured for 60 minutes at 20°C in those oocytes having undergone GVBD. GVBD was verified in oocytes incubated in parallel by fixation in 10% (w/v) trichloroacetic acid and dissection. Transport velocities are expressed as pmol·oocyte^{-1}·h^{-1}. (N.S. = not statistically significant)

TABLE 3-2. Measurement of saturable, Na^+-independent amino acid transport in oocytes folowing induction of GVBD by progesterone or TPA.

Substrate	Velocity Control	GVBD	% Decrease	P<
Progesterone:				
Thr	12.5 ± 0.7	10.5 ± 1.2	16.0	0.025
Leu	17.4 ± 4.5	6.3 ± 1.1	63.8	0.005
TPA:				
Thr	14.4 ± 4.4	3.1 ± 2.9	78.3	0.005
Leu	13.8 ± 3.9	1.8 ± 1.8	91.5	0.005

Germinal vesicle breakdown (GVBD) was induced in oocytes by the inclusion of progesterone (3.2 μM) or TPA (0.6 μM) in the surrounding medium and incubating for 10 to 12 hours as described in the Materials and Methods section. The Na^+-independent transport of 50 μM [^{3}H]threonine or [^{3}H]leucine was then measured for 60 minutes at 20°C in those oocytes having undergone GVBD. Saturable transport velocities were calculated by subtracting the velocities observed in the presence of 5 mM unlabeled substrate from those in the absence of excess substrate. GVBD was verified in oocytes incubated in parallel by fixation in 10% (w/v) trichloroacetic acid and dissection. Transport velocities are expressed as pmol$\cdot$ oocyte$^{-1}\cdot$h^{-1}.

magnitude than the decrease seen following TPA-induced GVBD (91.5%).

Discussion

Previous studies investigating the effects of GVBD on the transport of small solute molecules (Belle' et al., 1976; Richter et al., 1984) have focused primarily on the net changes in transport rather than on maturation-induced changes of specific carriers. This chapter describes an investigation into the effects of progesterone- or TPA-induced GVBD on four distinct transport activities. The activities studied were shown in Chapter I to be similar in many respects to Systems ASC, $B^{0,+}$, L, and asc. Maturation-induced alterations in the rates of substrate transport attributable to these carriers were investigated by measuring the Na^+-dependent AIB and threonine transport (Systems $B^{0,+}$ and ASC), and the Na^+-independent, saturable transport of threonine (System asc) and leucine (System L).

In agreement with other laboratories (Belle' et al., 1976; Richter et al., 1984), the experiments just described demonstrate a marked decrease in oocyte plasma membrane amino acid transport activity following GVBD. Although the physiological reason for this decrease in transport activity is as yet unknown, it has been theorized that this generalized decline serves to protect the oocyte from unbridled loss of soluble cytoplasmic constituents via carrier-mediated efflux (Van Winkle, 1988). Inherent in this

notion is the assumption that oocytes do, in fact, lose solute molecules to the surrounding medium through plasma membrane transporters. However, data reported in 1976 by Bravo et al. indicate that efflux may not be a problem for defolliculated oocytes. In this investigation, manually-defolliculated oocytes were assayed for soluble amino acid content before and after a 24-hour incubation in amino acid free medium. The results show a net decrease in the intra-oocyte concentration of only 4 (lysine, glutamic acid, valine, and leucine) of the 17 amino acids tested (Bravo et al., 1976). Therefore, the decline in transport activity seen following meiotic maturation may not serve to curtail efflux but may simply be a reflection of the fact that oocytes have no need for plasma membrane transporters while in the nutrient-poor pond water. Normal, or accelerated, turnover of existing transporters, subsequent to cessation of the translation of new carriers, could account for the observed decrease in transport activity. A report in 1981 by Kado et al. has shown that the total plasma membrane surface area of the oocyte decreases following progesterone-induced GVBD. Although the reason for this reduction in suface area is not known, it is plausible that the decrease is a reflection of enhanced internalization of existing plasma membrane proteins. Besides eliminating transporters that are no longer of use, the metabolites resulting from their proteolytic degradation could serve to supplement the

oocyte's yolk stores as a source of energy and amino acids to be used following fertilization. Further support for the endocytotic removal of plasma membrane transporters could be gathered by investigating the changes in hormone binding site number brought about by the addition of progesterone or TPA to the incubation medium. For example, estimation of the size of the oocyte insulin receptor population could be obtained by Scatchard analysis of [^{125}I]insulin binding as described by Shimizu et al. (1980). Unfortunately, the relative affinities of the substrate amino acids used in the preceding study for their respective carriers are not sufficiently high to allow for direct estimation of transporter number via binding assays.

Whatever the mechanism by which the oocyte brings about the reduction in the uptake of amino acids following GVBD, it is evident that both progesterone and TPA are effective in inducing the change. As outlined in the introduction to this chapter, incubation of stage 5 or 6 oocytes in progesterone-containing medium leads to a rapid decline in the cytoplasmic level of cAMP. This is followed by intra-oocyte alkalinization, increased protein phosphorylation, and, lastly, GVBD (Wasserman et al., 1986). However, the initial event in TPA-induced maturation is activation of protein kinase C (Stith and Maller, 1987), presumably due to the similarity in structure between diacylglycerol, a product of phosphoinositide breakdown, and the phorbol ester

(Nishizuka, 1984). Further, insulin, but not progesterone, has been shown to lead to phosphoinositide breakdown (Stith and Maller, 1987) and, hence, diacylglycerol production in oocytes. Given this dissimilarity in the initial event of progesterone- and TPA- (or insulin-)induced meiotic maturation, there exists the possibility of parallel differences in the response of amino acid transport activity to maturation. The data in Table 3-2 indicate that this may, in fact, be the case. Progesterone-induced GVBD caused a reduction of 16 and 63.8% in the saturable, Na^+-independent transport of threonine and leucine, respectively. The decrease seen for threonine and leucine following TPA-induced GVBD were 78.3% for the former and 91.5% for the latter. In all of these experiments, the non-saturable, or diffusion-mediated, Na^+-independent component of threonine and leucine transport was also seen to decline following GVBD (data not shown). The decrease in the non-saturable transport was observed whether TPA or progesterone was used although the decrease was of lesser magnitude when TPA was used. Non-saturable threonine and leucine transport declined 67 and 75% in the presence of progesterone but only 45 and 25%, respectively, in the presence of TPA. Although it is possible that these changes in membrane permeability are caused by the solvents used in the progesterone and TPA stock solutions, it is unlikely given the data in Fig. 3-3. For this experiment, progesterone was dissolved in ethanol or

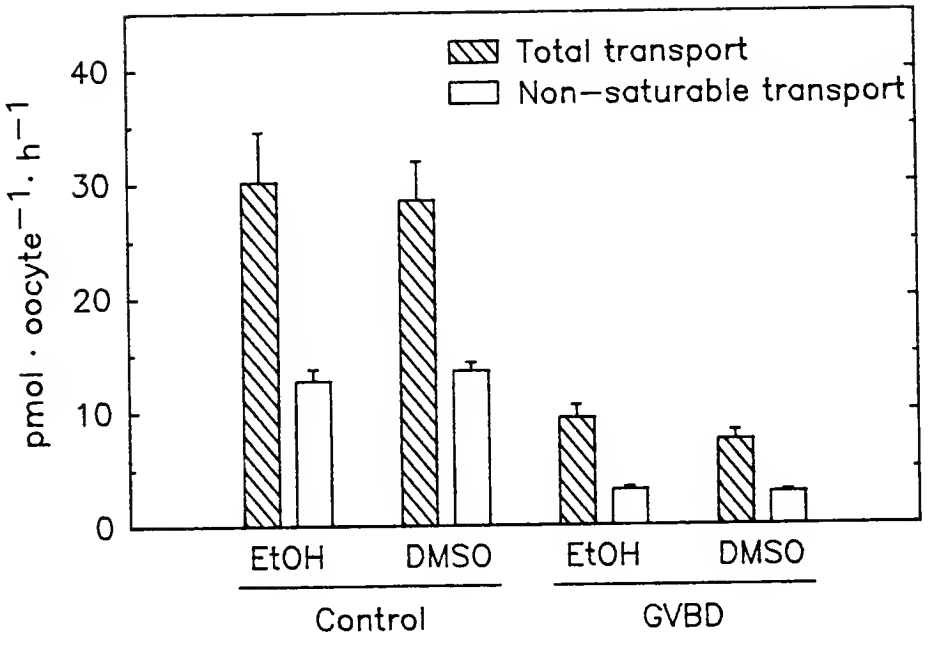

Figure 3-3. Effect of solvent, in the presence or absence of progesterone, on the saturable, Na^+-independent transport of leucine. Progesterone was dissolved in 95% (v/v) ethanol or DMSO at a concentration of 2 mg/ml. Oocytes were then incubated in MBM containing ethanol alone (0.05% (v/v)), DMSO alone (0.05% (v/v)), ethanol (0.05% (v/v)) plus progesterone (3.2 μM), or DMSO (0.05% (v/v)) plus progesterone (3.2 μM). After 10 hours of incubation at 20°C, those oocytes exhibiting a white spot at the animal pole were assayed for Na^+-independent leucine transport. Leucine transport was measured at 50 μM substrate concentration in the presence (Non-saturable transport) or absence (Total transport) of 5 mM unlabeled leucine as described in the Materials and Methods section.

DMSO prior to dilution into the incubation media. Following verification of GVBD, the saturable, Na^+-independent transport of leucine was determined as described in the Materials and Methods section. No difference between the effects of ethanol and DMSO, either alone or in conjunction with progesterone, was detectable (Fig. 3-3). Therefore, it is possible that the alterations in membrane permeability, as evidenced by changes in the rate of diffusion-mediated leucine uptake, are due to dissimilar effects of TPA and progesterone on the oocyte plasma membrane.

In contrast to the changes seen in transport rates measured in the absence of sodium, decreases in the Na^+-dependent transport of threonine caused by progesterone or TPA were essentially equal (Table 3-1). Although a decline in Na^+-dependent AIB transport was also observed, the change was found to be non-significant at the $P<0.05$ level. The lack of statistical significance is more likely a reflection of the extremely low AIB transport velocities, in combination with comparatively large standard deviation values, rather than a refractoriness of the oocyte AIB transporter to undergo a decline in activity in response to meiotic maturation.

In summary, the Na^+-dependent transport of threonine, and the saturable, Na^+-independent transport of threonine and leucine were shown to decrease markedly following induction of GVBD by progesterone or TPA. Differences in the magnitude

of the reduction in the saturable, Na^+-independent, and non-saturable components of threonine and leucine transport caused by progesterone and TPA were noted. No such differences between the effects of progesterone- and TPA-mediated GVBD on Na^+-dependent threonine transport were seen.

CHAPTER IV

TRANSLATION OF RAT LIVER mRNA IN *XENOPUS LAEVIS* OOCYTES

Introduction

Since its first mention in the literature (Lane *et al.*, 1971; Gurdon *et al.*, 1971), the use of frog oocytes as a means to study the metabolism and translation of exogenously-derived mRNA has steadily increased. The basic techniques involved, including the *in vitro* handling, incubation, and microinjection of the oocytes, have remained essentially unchanged since the early 1970's. Pioneering experiments employing the *Xenopus laevis* oocyte as an "*in vitro*" translation system often utilized relatively abundant or easily-isolated mRNA species. Examples include the mRNA's coding for rabbit globin (Lane *et al.*, 1971), duck globin (Lane *et al.*, 1973), and calf lens αA2 crystallin (Berns *et al.*, 1972). Further, the translation of viral polypeptides in *Xenopus laevis* oocytes was demonstrated in 1972 by Laskey *et al.* using encephalomyocarditis RNA. Proper post-translational modification of foreign proteins has been demonstrated repeatedly. Glycosylation (Colman *et al.*, 1981), signal sequence removal (Lane *et al.*, 1981), phosphorylation (Gedamu *et al.*, 1978), and multi-subunit protein assembly (Sumikawa *et al.*, 1981) are just a few of

the cases in which the Xenopus oocyte has faithfully translated and processed foreign proteins.

Accurate post-translational targeting of foreign proteins by the frog oocyte is further evidenced by the correct membrane insertion of functional ion channels (Sigel, 1987) and plasma membrane hormone receptors (Williams et al., 1988) following the microinjection of mRNA extracted from chick leg muscle or AR42J pancreatic cells in culture, respectively. Utilization of the oocyte system as an assay for a specific mRNA was demonstrated by Hirono et al. (1985) with the identification of a specific size fraction of mRNA capable of supporting the synthesis of rat brain Na^+-channels in Xenopus oocytes. More recently, it has been demonstrated that the oocyte system can be used as a functional screen in the molecular cloning of the cDNA for a specific protein. A case in point is the cloning of the intestinal Na^+/glucose co-transporter (Hediger et al., 1987a). Messenger RNA extracted from rabbit small intestine was size-fractionated via agarose gel electrophoresis and individual fractions microinjected into Xenopus laevis oocytes. After a 3-day incubation, the injected oocytes were assayed for the presence of the transporter. The size fraction of mRNA resulting in the most significant increase in oocyte Na^+-dependent glucose transport was then used to create a cDNA library. Messenger RNA transcribed in vitro from the library was then injected into oocytes which were

subsequently assayed for Na^+-dependent glucose transport. Identification of the cDNA for the transporter was achieved by injecting mRNA transcribed from increasingly smaller pools of cDNA.

Research in our laboratory has, for the past several years, focused on the System A amino acid transporter (Kilberg et al., 1986; Chiles et al., 1988; Fafournoux et al., 1989). As is the case with most other amino acid transporters, the isolation and purification of the System A carrier has proven to be an exceedingly difficult task. Our laboratory is currently pursuing several diverse routes in attempts to isolate the carrier. One of these, which has been the major focus of my thesis, involves the use of the Xenopus laevis oocyte as a functional screen to identify the mRNA coding for the System A carrier. The method is modeled after that utilized in the cloning of the intestinal Na^+-dependent glucose transporter described above (Hediger et al., 1987a).

Characterization of the endogenous oocyte amino acid transport activities, a necessary prerequisite to studies involving the identification of an exogenous carrier in the oocyte following microinjection of foreign mRNA, has been described in Chapter II. Although the capability of amino acid uptake assays in oocytes to distinguish among several different amino acid transport activities was illustrated in these initial experiments, it was not known if the assays

were of sufficient sensitivity to detect subtle changes in amino acid uptake rates subsequent to mRNA injections. As described in Chapter II, the principle means by which the presence of the System A carrier is documented in any cell or tissue, including oocytes, is via measurement of the Na^+-dependent transport of MeAIB or AIB. Direct monitoring of the carrier protein, or the mRNA coding for the protein, is not possible due to the fact that neither cDNA clones nor antibodies directed against the carrier exist. Therefore, the question of the stability of the System A mRNA or protein in the oocyte could not be answered. For these reasons, two additional proteins, for which both cDNA clones and antisera are available, were chosen to be monitored directly. The proteins selected were rat serum albumin (RSA) and phosphoenolpyruvate carboxykinase (PEPCK). An investigation of the expression of these two proteins will allow our laboratory to optimize the oocyte system for expression of rat liver mRNA.

Rat serum albumin, like the System A protein, is translated by membrane-bound ribosomes. Experiments described by Teraoka et al. (1982) have demonstrated that a related protein, mouse serum albumin, is synthesized in oocytes following the microinjection of mouse liver mRNA. Further, the protein is secreted into the surrounding medium by the oocyte and appears to be of identical molecular weight to authentic mouse serum albumin (Teraoka et al., 1982). A

report in 1981 by Richter and Smith, however, has indicated that the oocyte may, in fact, have a relatively limited capacity to handle mRNAs normally translated by membrane-bound ribosomes, such as that coding for serum albumin or the System A carrier. By monitoring the synthesis of RSA in the oocyte following the injection of rat liver mRNA, it should be possible to gauge the efficiency of the oocyte protein-translating machinery which would also be responsible for the translation of the System A protein.

Phosphoenolpyruvate carboxykinase, on the other hand, was chosen to be monitored in the oocyte following the microinjection of mRNA because of the similarities in regulation between it and System A (Kilberg et al., 1985; Meisner et al., 1983). The injection of glucagon or N^6,O^2-dibutyryl cyclic AMP (dibutyryl cAMP) into rats has been shown to result in an 8-fold stimulation of liver PEPCK enzyme activity and mRNA level in less than 2 hours (Cimbala et al., 1981; Iynedjian and Hanson, 1977). In an analogous manner, System A activity in primary cultures of rat hepatocytes has been shown to increase approximately 3-fold when measured 2 hours after exposure to glucagon (Barber et al., 1983). Under these conditions, System A activity continues to increase for an additional 4 hours to a level 5 times that of control (Barber et al., 1983). However, as mentioned above, direct measurements of the level of hepatic System A mRNA are not possible. Indirect evidence, based on

experiments utilizing RNA polymerase inhibitors has, however, demonstrated that essentially all of the increase in System A activity can be attributed to elevated mRNA production (Christensen and Kilberg, 1987).

In addition to similarities in the regulation of PEPCK and System A, the mRNAs coding for these proteins appear to have very short half-lives *in vivo*. The half-life of PEPCK mRNA has been estimated to be 40 minutes (Cimbala *et al.*, 1982; Nelson *et al.*, 1980). Similarly, the half-time for the decay of System A activity in hepatocytes isolated from a glucagon-injected rat is approximately 1.5 hours (Handlogten and Kilberg, 1984). Because the System A value was calculated from transport data and not mRNA levels, it is not known whether the decay reflects rapid mRNA turnover as well as protein degradation. However, because the glucagon-induced elevation in System A activity involves, presumably, mRNA synthesis, it can be argued that the rapid decay in activity would be likely to include mRNA turnover as well.

This chapter describes the synthesis in oocytes of 3 distinct proteins, RSA, PEPCK, and the System A carrier, subsequent to the microinjection of rat liver mRNA. Oocyte production of two of the proteins, RSA and PEPCK, has been monitored via immunoblotting techniques. Synthesis of the rat liver System A carrier, on the other hand, has been followed by measuring changes in the rate of oocyte Na^+-dependent AIB uptake. The degradation in the oocyte of the

mRNAs coding for RSA and PEPCK has been monitored by Northern blot analysis. Experiments involving variations in the amount and type (i.e. extracted from fed, glucagon-treated, or diabetic rat liver) of mRNA injected, as well as the length of the post-injection incubation period are described.

Materials and Methods

Isolation of Oocytes

Methodology for the isolation of oocytes is included in the Materials and Methods section in Chapter II.

Hepatoma Cell Culture

Rat hepatoma cells, from the Fao cell line, were grown in 75 cm^2 culture flasks (Falcon 3023) at 37°C in a humidified atmosphere of 5% (v/v) CO_2 and 95% (v/v) air. The cells were maintained in minimal essential medium (MEM), pH 7.4, supplemented with 25 mM sodium bicarbonate, 2.5 mM L-glutamine, 10 μg/ml penicillin, 5 μg/ml streptomycin, 28.5 μg/ml gentamicin, 0.2% (w/v) bovine serum albumin (BSA), and 5% (v/v) fetal bovine serum (FBS).

The rat Fao cell line was derived from rat H4 hepatoma cells after exposure to 8-azaguanine (Deschatrette and Weiss, 1974). The rat H4 cell line was derived from the Reuber H35 hepatoma (Reuber, 1961) which was in turn obtained by feeding rats a diet supplemented with N-2-fluorenyl-diacetamide, resulting in a bile-secreting, transplantable hepatocellular carcinoma. The Fao cell line is a well-

differentiated cloned cell line expressing a number of liver-specific proteins. Hormonal induction of tyrosine and alanine aminotransferases is also observed (Deschatrette and Weiss, 1974).

RNA Extraction from Cultured Cells

Total RNA was extracted from Fao cells using the method of Chirgwin et al. (1979) as modified by Maniatis et al. (1982). Cells were allowed to grow to confluency and were then rinsed twice with phosphate-buffered saline (PBS: 10 mM sodium phosphate, pH 7.4, 154 mM NaCl). The cells were removed from the surface of the flasks by trypsinization. This was done by the addition of 1 ml of a solution containing 0.05% (w/v) trypsin and 0.6 mM ethylenediaminetetraacetic acid (EDTA) in PBS to the flasks which were then incubated at 37°C for a few minutes. Detached cells were rinsed from the flasks with 5 to 10 ml PBS. Cells from 5 to 10 flasks were washed by centrifugation (500 x g for 5 minutes at 4°C in a swinging bucket clinical centrifuge) and resuspension in PBS, and lysed by vortexing the cell pellet in 5 ml guanidinium thiocyanate (GuSCN) solution containing 4 M GuSCN, 0.5% (w/v) sodium lauroyl sarcosine, 25 mM sodium citrate, 100 mM 2-mercaptoethanol, and 0.1% (v/v) Antifoam A (Sigma). The solution was transferred to a glass mortor and homogenized using a motor-driven Teflon pestle. The homogenate was then transferred to a 15 ml plastic centrifuge tube. The tube was centrifuged at

10,000 x g at 4°C for 10 minutes in a Sorvall SM-24 rotor. The supernatant fraction was layered over 1.25 ml of a 5.7 M CsCl cushion containing 25 mM sodium acetate, pH 5, and centrifuged at 36,000 x g at 20°C for 19 to 21 hours in an Beckman SW50.1 swinging bucket rotor. Before use, the CsCl solution was treated overnight with 0.2% (v/v) diethylpyrocarbonate (DEPC) and then heated at 65°C for at least 1 hour to eliminate residual DEPC. Following centrifugation, the resulting RNA pellet was dissolved in 0.4 ml of solubilization buffer containing 10 mM Tris, pH 7.5, 1 mM EDTA, 5% (w/v) sodium lauroyl sarcosine, and 5% (v/v) phenol (added just before use) and transferred to a clean polypropylene 50 ml Oak Ridge tube. The volume was brought to 5 ml with solubilization buffer, vortexed hard, and 4 M NaCl added to a final concentration of 0.1 M. Ten milliliters of a mixture of phenol, chloroform, and isoamyl alcohol (in the ratio of 50:49:1) were added, the tube shaken, and then centrifuged at 12,000 x g at 4°C for 10 minutes in a Sorvall SS-34 rotor. The aqueous layer was transferred to a clean tube and 2 ml solubilization buffer, minus phenol but including 0.1 M NaCl, was added to the organic phase. After shaking and centrifuging as above, the aqueous phases from the two extractions were combined and the RNA contained therein precipitated by the addition of one-tenth volume 2 M sodium acetate, pH 5.5 and three volumes absolute ethanol followed by incubation overnight at -20°C .

The RNA was sedimented by centrifugation and the pellet washed two times by resuspension in 75% (v/v) ethanol. The pellet was broken up and suspended in 1 ml of 0.4 M sodium acetate and precipitated with 2.5 volumes absolute ethanol. After sedimentation, the pellet was again washed with 75% (v/v) ethanol, dried under vacuum, and solubilized in DEPC-treated distilled, deionized water (ddH_2O). Following centrifugation at 20,000 x g at 10°C for 10 minutes in a Sorvall SS-34 rotor, the supernatant fraction was made 0.2 M in sodium acetate and precipitated with 2.5 volumes absolute ethanol. The RNA was sedimented, washed with 75% (v/v) ethanol, dried, and brought up in DEPC-treated ddH_2O. The numerous washes and precipitations serve to remove residual CsCl from the extracted RNA.

Total RNA extracted from CHO-K1, ala^{r}4-H2.1, and ala^{r}4-H3.9 cells was the generous gift of Dr. Ellis Englesberg. Poly(A)$^+$ mRNA was isolated from the total RNA by oligo(dT) cellulose column chromatography as described below. The parent cell line, CHO-K1, is a proline auxotroph whose growth can be inhibited by amino acids that prevent the uptake of proline (Moffett et al., 1983; Moffett et al., 1988). By selecting cells for growth in media containing increasingly higher concentrations of alanine, an amino acid known to competitively inhibit proline transport, mutant cells were developed which exhibit elevated proline transport. Two of these mutants, ala^{r}4-H2.1 and ala^{r}4-H3.9, display increased

rates of proline transport through the System A carrier, a transport system whose substrate specificity includes both proline and alanine. The H2.1 and H3.9 mutants were found to have System A-mediated proline uptake 18- and 29-fold higher, respectively, than the parent cells (Moffett et al., 1988).

RNA Extraction from Rat Liver

Total RNA was extracted from the livers of rats that had been treated as follows: "Control" rats were fed ad libitum and then given glucose by gastric gavage (5 g/kg body weight) 2.5 hours before killing. "Glucagon" rats were fasted for 24 hours, injected intraperitoneally with 1 mg glucagon, and killed 2.5 hours later. The glucagon was delivered in a 50:50 mix of PBS and ethanol (0.3 ml total volume). Diabetes was induced in rats by the intraperitoneal injection of 10 mg streptozotocin/100 g body weight. The streptozotocin was dissolved in 50 mM sodium citrate, pH 4.3. Urine glucose was monitored daily with Tes-Tape (Lilly) and blood glucose levels were measured in samples taken at the time of sacrifice (Sigma Procedure No. 635), generally 2 to 3 days following the injection of streptozotocin.

Rat liver RNA was extracted using the method of Chomczynski and Sacchi (1987) from male Sprague-Dawley rats (approximately 200 g body weight) that had been maintained on standard laboratory rat chow. After anesthesia, the liver was perfused free of blood with ice-cold PBS according to the method of Kilberg (1989). The liver was removed, weighed,

and then minced prior to homogenization at room temperature in solution D (4 M guanidinium thiocyanate, 0.5% (w/v) sodium lauroylsarcosine, 100 mM 2-mercaptoethanol, 25 mM sodium citrate, pH 7) using a motor-driven Teflon pestle. Solution D was used in the ratio of 8 to 10 ml per gram liver. The homogenate was then made 0.2 M in sodium acetate, pH 4, and extracted with an equal volume of water-saturated phenol and one-fifth volume of a mixture of chloroform and isoamyl alcohol (49:1). The mixture was placed on ice for approximately 15 minutes, after which time the aqueous and organic phases were separated by centrifugation at 12,000 x g for 20 minutes at 4°C in a Sorvall SS-34 rotor. Total RNA was precipitated from the aqueous phase by the addition of an equal volume of ice-cold isopropanol and chilling at -20°C for at least one hour. Sedimentation of the RNA was achieved by centrifugation at 12,000 x g for 20 minutes at 4°C. The resulting pellet was then solubilized in solution D and 2 volumes of ice cold absolute ethanol were added. The RNA was allowed to precipitate overnight at -20°C and was then sedimented as above. The pellet was washed twice with ice-cold 75% (v/v) ethanol, dried under vacuum, and solubilized in ddH_2O that had been treated with 0.1% (v/v) diethylpyrocarbonate (DEPC) and autoclaved.

RNA Extraction from Oocytes

In experiments designed to gauge the relative half-lives of various rat liver mRNA's in oocytes following

microinjection (described in a separate section), total RNA was extracted from oocytes using a modified version of the RNA extraction protocol described above. Typically, 10 oocytes were homogenized in 0.6 ml solution D and the homogenate centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatant fraction was transferred to a clean tube and extracted with phenol and chloroform as described previously. After separation of the aqueous and organic phases by centrifugation, the aqueous phase was extracted a second time with one-half the original volumes of phenol and chloroform. The RNA present in the aqueous phase was precipitated with an equal volume of isopropanol and the resulting pellet solubilized in 0.3 ml solution D. The remainder of the extraction is identical to that described for rat liver.

DNA Assay

Because determinations of relative quantities of PEPCK and RSA mRNA in total rat liver RNA depend, in part, on the precise quantity of RNA analyzed, precautions were taken to verify the absence of DNA, a potential source of interference in the measurement of RNA concentration. To test for the presence of DNA in the RNA preparations extracted from rat liver by the GuSCN procedure, the diphenylamine (DPA) reaction, described by Burton (1956), was used. Samples of total rat liver RNA were dissolved in 0.5 ml DEPC-treated ddH_2O and 0.5 ml perchloric acid added to each sample. The

samples were then heated in 4 ml polycarbonate centrifuge tubes at 70°C for 15 minutes, cooled, 2 ml DPA reagent added, and the tubes incubated overnight at room temperature. The DPA reagent was made by adding 0.1 ml of a 16 mg/ml solution of acetaldehyde to 20 ml of a DPA stock solution. The stock solution was made by dissolving 1.5 g DPA in 100 ml glacial acetic acid followed by the addition of 1.5 ml concentrated sulfuric acid. The stock solution was stored at room temperature in a brown glass bottle. After the overnight incubation, the absorbance at 600 nm of each sample was compared to that of DNA standards treated in an identical manner. The standards were prepared from salmon testes DNA. The results of this assay indicate that no more than 0.006 mg DNA/mg RNA was present in the RNA extracted from rat liver by the method described.

Determination of In Vivo System A Activity

Because the System A amino acid transporter and phosphoenolpyruvate carboxykinase (PEPCK) respond in parallel to changes in the metabolic state of the animal, the *in vivo* monitoring of the rat liver System A carrier allows one to estimate the status of PEPCK as well. The *in vivo* determination of rat liver System A activity in fed, glucagon-treated, and diabetic rats to be used as RNA donors was carried out according to the method of Kilberg and Neuhaus (1975) as follows: Approximately 1 hour prior to anesthesia, 2 μCi of [^{3}H]AIB (8 to 10 Ci/mmol) was injected

into the tail vein of the rat. Samples of blood (2 to 3 ml) and perfused liver (0.5 to 1.0 g) were taken and stored on ice while total RNA was extracted from the remainder of the liver as described above. The liver sample was then weighed, homogenized in 2 ml normal saline (154 mM NaCl), and the proteins precipitated from the homogenate with the addition of an equal volume of 10% trichloroacetic acid. After centrifugation at 12,000 x g for 15 minutes at 4°C, the radioactivity contained in a portion, typically 0.2 ml, was determined via liquid scintillation spectrophotometry. The blood sample was allowed to clot and the resulting serum was treated in manner equivalent to that for liver. The quantity of radioactivity present in 1 g liver divided by that present in 1 ml serum is referred to as the distribution ratio (DR). When compared among the fed, diabetic, and glucagon-treated conditions, the DR can be used to gauge the relative degree of glucagon-dependent induction of System A and, presumably, PEPCK in the rat liver. Following the RNA extraction, Northern analysis is used to verify the augmentation in PEPCK mRNA level.

Oligo(dT) Cellulose Column Chromatography

Poly(A)$^+$ mRNA was purified by oligo(dT) cellulose (Sigma) column chromatography. Oligo(dT) cellulose chromatography columns were made by first washing 0.25 g oligo dT cellulose in a sterile plastic 12 ml tube using DEPC-treated ddH_2O. The tube was gently rotated end-over-

end by hand and the cellulose allowed to sediment under unit gravity. The supernatant fraction and fines were poured off and the wash repeated. The washed cellulose was then resuspended in fresh DEPC-treated ddH_2O and the slurry poured into a disposable plastic chromatography column (BioRad no. 731-1550) that had previously been rinsed with DEPC-treated ddH_2O. The ddH_2O was allowed to flow through and the column equilibrated with at least 10 column volumes of DEPC-treated RNA loading buffer (LB) containing 0.5 M NaCl, 10 mM Tris, pH 7.4. The Tris was added after the buffer had been treated with DEPC and autoclaved due to the incompatability of DEPC and this compound. An equal volume of RNA diluting buffer (DB), containing 1 M NaCl, 20 mM Tris, pH 7.4, was added to the RNA solution consisting of total RNA in DEPC-treated ddH_2O. Best results were obtained with final RNA concentrations of not more than 1 mg/ml as higher concentrations sometimes resulted in very slow flow rates through the column. The solution was mixed and heated at 65°C for 3 minutes and immediately cooled on ice. The solution was then poured over the column, allowed to flow through, and non-binding (i.e. poly(A)$^-$) RNA washed through with 5 to 10 column volumes of LB. The poly(A)$^+$ mRNA was eluted with DEPC-treated ddH_2O. Fractions enriched in mRNA were pooled, divided into aliquots of approximately 30 μg nucleic acid each, made 0.3 M in sodium acetate, and precipitated overnight with ethanol. Following

sedimentation, the pellets were washed twice by resuspension in ice-cold 75% ethanol and stored at -70°C under 75% (v/v) ethanol.

Microinjection

Messenger RNA to be microinjected into oocytes was first dissolved in DEPC-treated ddH_2O and then centrifuged at 12,000 x g at 4°C for a few seconds immediately prior to use. This centrifugation step was included in order to ensure that the mRNA solution was free of particulate matter that could clog the microinjection needle. Microinjections were performed using a Hamilton Micro Lab P microprocessor-controlled pipettor programmed to deliver 40 nl/injection (Hitchcock and Friedman 1980; Hitchcock et al. 1987). Microinjection needles were pulled from 2 μl Drummond "MICROCAPS" using a Narishige PN-3 micro-pipette puller. Following microinjection, all incubations were carried out in MBM at 18-20°C with daily changes of medium.

Oocyte Homogenization, SDS-polyacrylamide Gel Electrophoresis, and Immunoblotting

Oocytes were homogenized in ice-cold buffer (20 to 40 μl buffer per oocyte) containing 100 mM NaCl, 1 mM phenylmethylsulfonylfluoride, 5 mM benzamidine, 1% (v/v) Triton X-100, and 20 mM Tris, pH 7.6 (Colman, 1984) using a motor-driven teflon pestle designed to fit in a standard 1.5 ml Eppendorf microcentrifuge tube. The homogenate was spun in a microcentrifuge at 12,000 x g for 10 minutes at 4°C and

the infranatant fraction, between the yolk platelet pellet and the lipid pellicle, was removed using a 25 gauge needle and stored at -70°C.

In experiments designed to investigate proteins secreted from the oocyte, incubation medium (i.e. MBM) was collected at specified times after microinjection. Immediately after collection, all media were made 1 mM in PMSF and 5 mM in benzamidine prior to storage at -70°C. Oocytes were typically incubated in 30 μl MBM per oocyte in order to maximize the concentration of secreted proteins in the medium. Utilization of smaller amounts of MBM per oocyte resulted in reduced oocyte viability.

Oocyte proteins contained in homogenate or medium samples were separated via sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis according to the method of Laemmli (1970). Briefly, oocyte proteins were solubilized and denatured by boiling for 2 to 5 minutes in sample dilution buffer (SDB) containing 1% (w/v) SDS, 30 μg/ml bromophenol blue, 12% (v/v) glycerol, 720 mM 2-mercaptoethanol, and 125 mM Tris, pH 6.8. The ratio of homogenate or medium to SDB could be as high as 1:1 without obvious deleterious effects. In some cases, however, the volume of protein sample (i.e. homogenate or medium) was too large to be loaded into the wells of the polyacrylamide gel. In these instances, proteins were first precipitated from homogenate or medium samples by the addition of 24% (w/v) TCA

to a final concentration of 10% (w/v) and incubation on ice for 30 to 60 minutes. The samples were then centrifuged at 12,000 x g for 15 minutes at 4°C and the pellet washed twice, with resuspension, in 0.75 ml ethyl ether to remove the TCA. Complete removal of TCA was crucial at this time in order to ensure solubility in SDB. The final pellet was allowed to air dry, SDB was added, and the solution vortexed thoroughly. After boiling for 2 to 5 minutes, the samples were allowed to cool to room temperature and were then centrifuged at 12,000 x g for 1 minute at 4°C before being loaded into the wells of a 7.5% (w/v) polyacrylamide separating gel equipped with a 4.5% (w/v) polyacrylamide stacking gel. Both the separating and stacking gels contained 0.1% (w/v) SDS. Electrophoresis was carried out in buffer containing 192 mM glycine, 25 mM Tris, pH 8.3, and 0.1% (w/v) SDS at 20 to 30 mA, constant current, until the bromophenol dye front was within 1 cm of the bottom of the gel.

For electroblotting, the gel was removed from the electrophoresis apparatus, the stacking gel was cut away and discarded, and the separating gel immediately placed into vacuum-degassed transfer buffer containing 192 mM glycine, 25 mM Tris-base, pH 8.3, and 20% (v/v) methanol (Towbin et al., 1979). After 15 minutes, a piece of Whatman 3MM chromatography paper, cut slightly larger than the gel, was maneuvered under the gel. The chromatography paper and the gel were then lifted together and placed on a square of

Scotch-Brite pad that had been soaked for at least 15 minutes in transfer buffer. Bubbles were removed from between the gel and the chromatography paper using a glass stir rod. The gel was then covered with a piece of nitrocellulose paper, cut to the size of the gel, that had been equilibrated for at least 15 minutes in transfer buffer. Bubbles were removed as above. A second piece of wet chromatography paper was then laid over the nitrocellulose paper, bubbles were removed, and a second wet Scotch-Brite pad was laid on top. The "sandwich", consisting of pad, chromatography paper, gel, nitrocellulose, chromatography paper, and pad, was then secured in a perforated plastic holding device and submerged vertically in a tank of transfer buffer. (The Scotch-Brite pads, plastic holding device, and tank were from BioRad.) Proteins contained in the gel were electrophoretically driven into the nitrocellulose paper by the application of 30 V, constant voltage, for 14 to 16 hours followed by 40 V for an additional 2 hours. The plastic holding device was then removed from the tank and disassembled. The nitrocellulose paper was removed and proteins adhering to it were visualized by staining with a 0.01% (w/v) amido black solution containing 50% (v/v) methanol and 10% (v/v) acetic acid. The blot was destained in 50% (v/v) methanol, 10% (v/v) acetic acid.

After destaining, blots that were not to be used immediately were placed, stain side up, on a clean glass

plate, covered with plastic wrap, and stored at -20°C. Otherwise, blots were prepared for immunoblotting by first rinsing twice, for about 30 seconds each rinse, in PBS containing 0.02% (w/v) sodium azide (PBS-azide). Blocking of exposed nitrocellulose was achieved by incubation in PBS-azide containing 5% (w/v) Carnation non-fat dry milk (blocking buffer) for 4 to 16 hours using 70 to 80 ml blocking buffer per blot. This and all subsequent immunoblotting incubations were carried out at room temperature with constant agitation. The blocking buffer was poured off and 50 to 60 ml fresh blocking buffer containing primary antibody (i.e. either immune or non-immune serum) was added and the incubation continued for an additional 2 hours. Antiserum against PEPCK was provided by Daryl K. Granner and that for RSA was purchased commercially. The exact quantity of serum to be used was determined empirically and is stated in the figure legends. Following this incubation, the blocking buffer containing serum was poured into a plastic 50 ml centrifuge tube and stored for further use at -20°C. The blot was rinsed 4 times for a few seconds each time with PBS-azide and then washed for 25 minutes in 70 to 80 ml PBS-azide containing 0.3% (v/v) polyoxyethylenesorbitan monolaurate (Tween 20). This was followed by 4 additional rinses with PBS-azide as above. After rinsing, the blot was incubated for 1 hour in 50 to 60 ml blocking buffer containing secondary antibody. Detection of primary

antibody was accomplished by one of two methods: colorimetric, using alkaline phosphatase-conjugated secondary antibody; or autoradiographic, using ^{125}I-coupled secondary antibody. Secondary antibodies, purchased from Sigma, were raised in donkeys using either sheep (for anti-PEPCK primary antibody) or goat (for anti-RSA primary antibody) IgG as antigen. Alkaline phosphatase-conjugated secondary antibody was used at a 1:1,000 dilution and ^{125}I-coupled secondary antibody was used at 10^6 cpm/ml blocking buffer. The procedure for coupling ^{125}I to IgG is described in a separate section below. Following incubation in secondary antibody, blocking buffer containing alkaline phosphatase-conjugated secondary antibody was discarded while that containing ^{125}I-coupled secondary antibody was stored at 4°C behind lead for further use. The blot was washed with PBS-azide, PBS-azide-Tween 20, and PBS-azide as described above. For immunoblots utilizing ^{125}I-coupled secondary antibody, the blot was placed on a dry paper towel and allowed to dry in air at room temperature. The dried blot was then placed between sheets of plastic wrap and allowed to expose X-ray film (Kodak X-OMAT AR) at room temperature for varying periods of time depending on the strength of the signal. For alkaline phosphatase-conjugated secondary antibody, the blot was rinsed 2 additional times for a few seconds each time with 100 mM Tris, ph 8.8 containing 1 mM $MgCl_2$ (Tris-$MgCl_2$). The colorimetric

substrate buffer was prepared by adding 5-bromo-4-chloro-3-indolyl phosphate (BCIP) from a 100 mg/ml stock solution in dimethylsulfoxide (DMSO) to Tris-$MgCl_2$ containing 0.1 mg/ml nitro blue tetrazolium (NBT). The final concentration of BCIP was 0.125 mg/ml. Immediately after the addition of BCIP, the colorimetric substrate buffer was poured over the blot and the colorizing reaction was allowed to continue in the absence of motion until specific bands had reached the desired intensity; often in as little as 2 to 5 minutes but in some cases as long as 2 hours. The reaction was terminated by pouring off the colorimetric substrate buffer and rinsing the blot 4 or 5 times with deionized water (dH_2O). The blot was then air dried on a paper towel, covered with plastic wrap and photographed using Polaroid Type 55 Land film. The negative was treated for 5 to 10 minutes in 18% (w/v) sodium sulfate and then rinsed with several changes of dH_2O for at least 30 minutes.

Radioiodination of Secondary Antibodies

Radioiodination of secondary antibodies was performed using the chloramine T method described by Greenwood et al. (1963). Briefly, 0.2 to 0.3 mg of either anti-sheep or anti-goat IgG (both from Sigma) were dissolved in 0.5 ml PBS-azide in a screw-cap 1.5 ml Eppendorf microcentrifuge tube. One millicurie of ^{125}I, in the form of sodium [^{125}I]iodide (13 to 14 mCi/μg iodine), was added to the tube followed by the addition of 10 μl of a chloramine T stock solution containing

2.5 mg chloramine T/ml PBS-azide. The tube was tightly capped and rotated end-over-end for 30 seconds. The reaction was stopped by the addition of 120 μg sodium metabisulphite from a 6 mg/ml stock solution in PBS-azide. Unincorporated radioactivity was removed using either a small, 5 to 8 ml bed volume, Sephadex G-100 column or a 1 ml Sephadex G-50 (fine) "spin column" as described below. Both the G-100 and G-50 were swollen in PBS-azide for at least 48 hours before use. The G-100 column was prepared in a thick-walled glass Pasteur pipette that had been plugged with a small quantity of glass wool. Approximately 2 to 3 bed volumes of PBS-azide were allowed to flow through the column which was then totally submerged in PBS-azide, to prevent drying, until use. Following the addition of the sodium metabisulphite, the reaction mixture was applied to the top of the column bed, allowed to flow into the bed, and then PBS-azide carefully applied to the column. Fractions (10 to 15) of 0.5 ml each were collected and the radioactivity contained in 10 μl aliquots of each fraction monitored using a Searle Model 1185 gamma counter. The fractions in the void volume containing the greatest amount of radioactivity were combined and stored in a lead container at 4°C. The spin column was prepared by filling a 1 ml disposable syringe, plugged with glass wool, with a slurry of Sephadex G-50 (fine) in PBS-azide and centrifuging at approximately 100 x g for 45 seconds in a table-top centrifuge equipped with a swinging-bucket rotor.

One-half milliliter PBS-azide was added to the packed bed which was then centrifuged again and the flow-through fraction discarded. The radioiodination reaction mixture was then added and the column centrifuged as before. The total flow-through fraction was collected and stored in a lead container at 4°C.

Agarose Gel Electrophoresis and Northern Analysis

RNA samples to be investigated via Northern analysis were first subjected to electrophoresis in a denaturing 1% (w/v) agarose gel containing formaldehyde. The gel was prepared by dissolving 1 g high-gelling-temperature agarose in 80 ml ddH_2O in a glass Erlenmeyer flask using a microwave oven. Dissolution of the agarose was facilitated by first stirring the agarose solution for 3 to 5 minutes using a magnetic stir plate. The flask containing the melted agarose was then placed in a 65°C water bath to facilitate cooling to this temperature. Formaldehyde (12 ml of a 37% (v/v) solution) and 10x 3-[N-morpholino]propanesulfonic acid (MOPS) buffer (10ml) containing 200 mM MOPS, pH between 5.5 and 7.0, 50 mM sodium acetate, and 10 mM EDTA were then added and the mixture swirled gently. The agarose solution was then poured into a 100 ml graduated cylinder and the volume made to 100 ml, if necessary, with ddH_2O. The solution was returned to the flask, swirled gently again, and poured into the slab gel mold and allowed to cool and solidify for at least 30 minutes. The well-forming comb was carefully

removed from the cooled gel which was then placed into the electrophoresis unit. Sufficient tank buffer, consisting of 1x MOPS buffer, was then poured into the unit until the top of the gel was approximately 0.5 to 1 cm under the surface. RNA samples stored as pellets under 75% (v/v) ethanol were dried under vacuum and dissolved, with heating at 65°C, in DEPC-treated ddH_2O. Ribonucleic acid was quantitated by measuring the absorbance of light at 260 nm wavelength (1 absorbance unit at 260 nm = 40 μg RNA/ml). Measured aliquots (5 to 30 μl) of the RNA solutions were then added to RNA loading buffer such that the final total volume was 60 μl per sample. Loading buffer was prepared by combining 0.72 ml deionized formamide, 0.16 ml 10x MOPS buffer, 0.26 ml 37% (v/v) formaldehyde, 0.18 ml ddH_2O, 0.1 ml 80% (v/v) glycerol, and 0.08 ml of a saturated solution of bromophenol blue in ddH_2O (Davis et al., 1986). Formamide was deionized by stirring for 30 minutes at 4°C with AG 501-X8 mixed-bed resin (Bio-Rad) using 5 g resin for 50 ml formamide. The solution was filtered twice through Whatman No. 1 filter paper, aliquoted, and stored at -70°C. The RNA in the loading bufer was denatured by heating at 65°C for 15 to 20 minutes and immediately cooled on ice. Following the addition of 1 μl of a 1 mg/ml solution of ethidium bromide (EtBr) in ddH_2O to each sample, the samples were mixed gently and loaded into the wells of the agarose gel. Electrophoresis was carried out at

a constant voltage of 30 V until the bromophenol blue had migrated three-quarters of the length of the gel.

The gel was removed from the electrophoresis unit and photographed under ultraviolet illumination using Kodak Type 55 or 57 film. Negatives (Type 55) were treated as previously described. For transfer of the RNA to hybridization membrane (i.e. Northern blot, based on the Dupont/NEN protocol), the gel was first cleared of formaldehyde by washing for 5 minutes in dH_2O with changes of water at 1 minute intervals. The gel was then slowly agitated for 20 minutes in 50 mM NaOH, rinsed twice with dH_2O, and incubated for 30 minutes in 100 mM Tris, pH 7.0. The gel was then laid, face down, on a fully wetted piece of Whatman 3MM chromatography paper, the ends of which were submerged in 10x SSPE (20x SSPE contains 3 M NaCl, 20 mM EDTA, and 200 mM sodium phosphate, pH 7.4). The gel was overlaid with GeneScreen (NEN) nylon hybridization membrane that had previously been hydrated in dH_2O for 1 minute and then soaked in 10x SSPE for 15 minutes. Bubbles were removed from between the gel and the GeneScreen by rolling a glass stir rod back and forth over the membrane. The GeneScreen was overlaid with two 10x SSPE-saturated sheets of 3MM paper followed by 2 dry sheets. A 10 to 15 cm stack of paper towels was laid on top and compressed slightly with a mass of approximately 400 g on a sheet of glass. Transfer of RNA to the hybridization membrane was verified the next day, usually

18 to 24 hours later, by inspection under ultraviolet illumination. The membrane was wrapped in plastic wrap and the RNA cross-linked to it by exposure to high intensity ultraviolet light for 3.5 minutes (Church and Gilbert, 1984). Prior to hybridization, the blot was washed free of adhering agarose by pouring boiling 20 mM Tris, pH 7, over it and agitating for 10 minutes at room temperature. The blot was further prepared by incubation in 0.1x SSC (20x SSC contains 3 M NaCl, 0.3 M sodium citrate, pH 7.0), 0.1% (w/v) SDS at 65°C for at least 1 hour. The blot was prehybridized in 0.5 M sodium phosphate, pH 7.2, 7% (w/v) SDS, 1% (w/v) BSA, 1 mM EDTA (hybridization buffer) at 65°C for at least 15 minutes prior to hybridization in the same buffer containing radiolabelled probe. Prehybridization for as long as 16 hours had no obvious deleterious effects. Twenty five to fifty nanograms of cDNA containing sequences complimentary to rat serum albumin (RSA), rat liver PEPCK, or mouse ß-actin mRNA were radiolabeled with ^{32}P using a Random Primers DNA Labeling Kit from BRL. The cDNA clone for RSA was provided by Tom Sargent, that for PEPCK by Richard Hanson, and that for ß-actin by Harry Nick. Following incubation, the probe mixture was extracted first with an equal volume of a 50:50 mixture of phenol and chloroform and then with an equal volume of chloroform alone. Incubations were generally carried out for 3 hours although equivalent results were obtained when the primer extension reaction was allowed to

proceed overnight. The amount of precipitable radioactivity in an aliquot of the extracted probe mixture was determined by precipitation in 10% (w/v) TCA followed by filtration, under vacuum, through a glass fiber filter disk. Nonprecipitable radioactivity was washed through the filter with 10 ml ethanol followed by 10 ml acetone. Total and precipitable radioactivity were quantitated via liquid scintillation spectrophotometry. Blots were hybridized at 65°C in 25 ml hybridization buffer containing approximately 1.5×10^6 precipitable cpm of ^{32}P per ml hybridization mixture. After 20 to 24 hours of hybridization, the blot was washed at room temperature with several changes of 2x SSC followed by several changes of 0.5x SSC at 65°C. All washes were for 4 to 5 minutes each. The washed blot was wrapped tightly in plastic wrap and allowed to expose X-ray film at room temperature or at -70°C depending on the amount of radioactivity on the blot. Blots containing large amounts of radioactivity (e.g. >20 counts per second per band) required less than one hour at room temperature to expose the film. On the other hand, blots containing very small amounts of radioactivity (e.g. <5 counts per second per band) were generally placed at -70°C in order to increase the response of the film to the decaying beta particles (Amersham Review No. 23). Scanning densitometry of serial dilutions of radiolabeled probe revealed no difference in the exposure

characteristics whether exposure was at room temperature or -70°C.

Blots to be probed additional times were first stripped by boiling in 0.1x SSC containing 1% (w/v) SDS for 30 to 45 minutes. The effectiveness of stripping was monitored by allowing the blot to expose X-ray film for 24 hours. The stripping procedure was repeated if bands were detected following the exposure period.

Scanning Densitometry

Quantitation of exposed areas of autoradiographs and fluorographs, or dark areas of Polaroid negatives, was by means of scanning densitometry using an LKB Ultroscan XL laser densitometer. Linearity of the X-ray film response as well as that of the densitometer was determined by scanning autoradiographs made from blots containing serial dilutions of radiolabeled probe. The loading of equal quantities of RNA on agarose gels, prior to Northern analysis, was verified initially by visual examination under ultraviolet illumination of ethidium bromide staining intensities for the ribosomal RNAs. When necessary, Polaroid negatives of the ethidium bromide stained gel were scanned and the relative intensities of the ribosomal RNA (rRNA) bands used to correct for RNA loading insufficiencies. Calculations based on scans of the 18s, 28s, or both rRNA bands gave equivalent results.

Results

Analysis of RNA

As described in the Introduction to this chapter, treatment of rats with glucagon leads to a rapid increase in the quantity of PEPCK mRNA in the liver (Cimbala et al., 1981; Iynedjian and Hanson et al., 1977). Further, the experimental induction of diabetes in rats by means of streptozotocin leads to an augmentation in PEPCK mRNA level equivalent to that seen after glucagon administration (Cimbala et al., 1982). The experimental data with regard to RSA mRNA levels, however, is not interpreted as easily. Early experiments had demonstrated a decline in albumin secretion from perfused rat liver (Tavill et al., 1973) or isolated parenchymal cells (Dich and Gluud, 1976) following the inclusion of glucagon in the perfusing buffer or culture medium. In other work, the withdrawal of insulin from diabetic rats was shown to bring about a 5-fold reduction in the rate of albumin secretion (Jefferson et al., 1983). Due to the fact that hyperglucagonemia is associated with the diabetic condition (Unger, 1976), the possibility exists for glucagon to be labeled as the cause of the decline in albumin secretion seen upon the withdrawal of insulin. However, because diabetes is a complex disease involving perturbations in a multiplicity of physiological processes, the potential also exists for a variety of hormones and/or metabolites to be involved in the regulation of albumin

metabolism. More recent work has demonstrated that this may, in fact, be the case. Utilizing data gathered from nuclear run-off assays, performed with isolated rat hepatocyte nuclei, Nawa et al. (1986) have shown that glucagon alone has no effect on the transcription rate of the albumin gene. On the other hand, dexamethasone was shown to be a powerful regulator, increasing the rate of albumin gene transcription 7-fold within 2 hours after application. Further, glucagon was shown to play a permissive role, enhancing the effect seen with dexamethasone. In an effort to avoid the complications inherent in vivo, the experiments performed by Nawa et al. were carried out using primary cultures of rat hepatocytes in the absence of serum and hormone supplements. In agreement with the in vitro data, experiments carried out in adrenalectomized rats by the same group imply that the expression of albumin is dependent upon glucocorticoids and that this dependence is modulated by other hormones. That this supposition is indeed the case is demonstrated by the presence of a glucocorticoid regulatory region 5' to the start of the rat albumin gene (Nawa et al., 1986). This region is highly similar to analogous regions in murine mammary tumor viruses (Scheidereit et al., 1983), human metallothionine (Karin et al., 1984), and human growth hormone genes (Moore et al., 1985; Slater et al., 1985). Therefore, it would appear that RSA mRNA levels in the livers of glucagon-treated rats must depend on factors such as the

nutritional status of the animal at the time of hormone injection as well as circulating levels of other hormones, growth factors, and metabolites.

As described in the Materials and Methods section, total RNA was extracted fron the livers of rats that had been either treated with glucagon, made diabetic with streptozotocin, or fed ad libitum and then force fed glucose by gastric intubation. In order to verify that the predicted alterations in the level of PEPCK mRNA had indeed occurred as a result of these manipulations, all RNA samples to be used for microinjection into oocytes were subjected to Northern analysis. All RNA samples were also examined using a RSA cDNA probe in order to examine the changes in levels of this mRNA resulting from the experimental conditions. One of the limitations of this analytical technique is that estimations of mRNA levels must be made relative to the total amount of RNA present, rather than on the absolute quantity of the specific mRNA in question. Therefore, it is crucial that the amount of each RNA sample loaded onto the agarose gel be the same for all conditions. Although an attempt was made in this and all subsequent experiments to load equal amounts of RNA into each well of the gel, differences were sometimes encountered. When necessary, disparities in the amount of RNA per sample were adjusted by means of two distinct methods: 1) quantitation of the relative amount of RNA present by densitometric analysis of photographic negatives

of the ethidium bromide-stained ribosomal RNA within each lane; or 2) expression of data relative to the amount of ß-actin mRNA present, as revealed by Northern analysis. The utilization of both methods will minimize the limitations inherent in each.

Figures 4-1 and 4-2 will serve to illustrate the techniques just described. Photographs of the ethidium bromide-stained agarose gels containing control (i.e. fed) and glucagon RNA, or control and diabetic RNA are shown in Fig.4-1. The negatives of these photographs were analyzed by laser scanning densitometry. The results of the densitometric analysis indicate that, based on the intensity of ethidium bromide staining of ribosomal RNA, the amount of RNA loaded into each pair of lanes differed by no more than 10%. The RNA contained in the agarose gels depicted in Fig. 4-1 was blotted to GeneScreen and probed with primer-extended ^{32}P-labelled cDNA probes for mouse ß-actin and rat liver PEPCK. The results are illustrated in Fig. 4-2. Data regarding relative quantities of PEPCK mRNA in the various RNA preparations are recorded in Table 4-1. Although some differences were encountered between the glucagon/control or diabetic/control ratios calculated using either the ethidium bromide or ß-actin data, the trends are similar regardless of the technique used to adjust for apparent inequalities in RNA quantity. Treatment of rats with glucagon resulted in a 4- to 5-fold increase in the quantity of PEPCK mRNA compared to

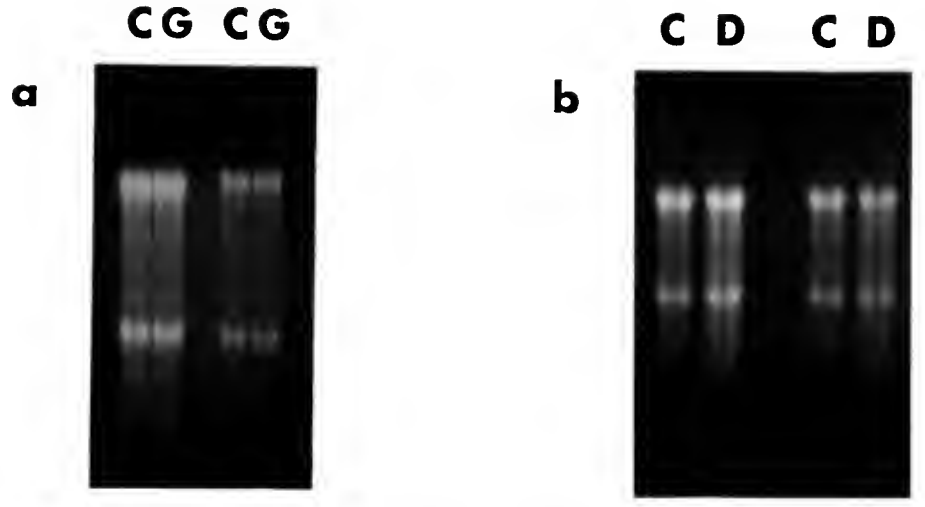

Figure 4-1. Ethidium bromide-stained agarose gels of rat liver RNA isolated from control, glucagon-treated, or diabetic animals. Ribonucleic acid was extracted from the livers of control (C), glucagon-treated (G), and diabetic (D) rat livers as described in the Materials and Methods section. Samples of the RNA were diluted in RNA loading buffer containing ethidium bromide and subjected to electrophoresis in a denaturing 1% (w/v) agarose gel. The gel was then photographed under ultraviolet illumination. For control and glucagon-treated RNA (a), 12 μg (left 2 lanes) or 30 μg (right 2 lanes) of RNA were loaded in each lane of the gel. For control and diabetic (b), 10 μg (left 2 lanes) or 15 μg (right 2 lanes) were loaded per lane. Negatives of the above photographs were used for 2-dimensional laser scanning densitometry. Scanning of the 28s, 18s, or both ribosomal RNA bands gave equivalent results. Scan data from the 28s band alone was used in this and all subsequent experiments to adjust for inequalities in the quantities of RNA in each lane.

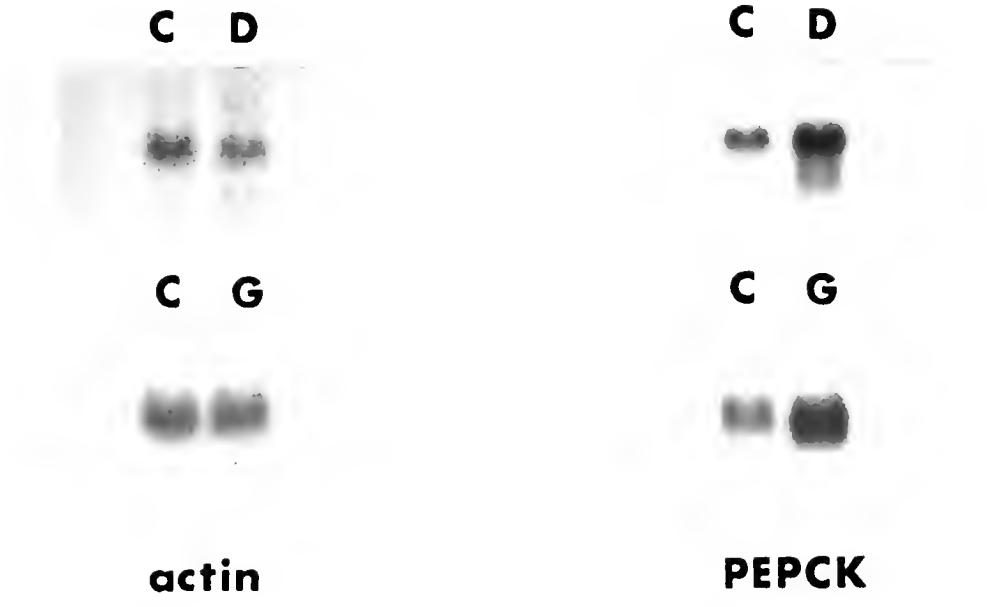

Figure 4-2. Results of Northern analysis of rat liver RNA extracted from control (C), glucagon-treated (G), or diabetic (D) animals. Ribonucleic acid contained in the gels pictured in Fig. 4-1 was transferred to Gene Screen as described in the Materials and Methods section. The blots were then hybridized with ^{32}P-labelled single-stranded cDNA probes for mouse ß-actin or rat liver PEPCK. Radioactivity adhering to the blots after washing in 0.5% (v/v) SSC at 65°C was allowed to expose X-ray film for varying amounts of time. The film was then developed and the exposed areas quantitated via 2-dimensional laser scanning densitometry.

Table 4-1. Determination of the relative concentration of PEPCK mRNA in total RNA extracted from the livers of control, glucagon-treated, or diabetic rats.

mRNA	Ratio[a]	EtBr[b]	ß-actin[c]
PEPCK	G/C	5.4 ± 1.6	4.3 ± 2.6
PEPCK	D/C	2.3 ± 0.1	3.3 ± 0.5

Total RNA was extracted from the livers of rats that had been fed ad libitum and then gavaged with glucose (5 g/kg body weight) 3 hours prior to anesthesia (control), injected with glucagon 2.5 hours prior to anesthesia, or made diabetic by the injection of streptozotocin 48 hours prior to anesthesia. One hour prior to anesthesia, all rats were injected into the tail vein with [^{3}H]AIB as described in the Materials and Methods section. The distribution ratios (i.e. the quantity of radioactivity present in 1 g of liver divided by that in 1 ml of serum) calculated for the 3 conditions were 2.5, 29.5, and 6.0 for control, glucagon-treated, and diabetic, respectively. Samples of RNA from the 3 conditions were subjected to agarose gel electrophoresis and the ethidium bromide (EtBr) staining pattern recorded by photography under ultraviolet light. The RNA was then transferred to GeneScreen and allowed to hybridize with ^{32}P-labelled single-stranded cDNA probes for PEPCK or mouse ß-actin.

a. Data are reported as follows:
   G/C = mRNA present in glucagon-treated rat liver relative to that in control rat liver.
   D/C = mRNA present in diabetic rat liver relative to that in control rat liver.

b. Ratios after RNA loading inequalities had been adjusted using ethidium bromide scanning data.

c. Ratios after RNA loading inequalities had been adjusted using ß-actin Northern data.

control. Similarly, induction of diabetes in rats caused a 2- to 3-fold increase in the mRNA coding for this enzyme.

The differences observed in the AIB distribution ratios (DR) among the control, glucagon-treated, and diabetic rats reflect changes in System A activity *in vivo* in response to the various experimental manipulations (Kilberg and Neuhaus, 1975). At the time of liver perfusion, the DR of AIB in glucagon-treated and diabetic rats was 29.5 and 6.0, respectively. However, in the control rat the value was 2.5. These data represent increases in System A activity of 11.8- and 2.4-fold over control for glucagon-treated and diabetic rats, respectively.

Immunological Analysis of Protein Synthesis in Oocytes

The synthesis in oocytes of PEPCK and RSA following the microinjection of rat liver mRNA was monitored by means of immunoblot analysis (Blake *et al.*, 1984; Johnson *et al.*, 1984). Initial testing of the antisera was performed using total cytosolic rat liver protein as the source of antigen. The protein mixture consisted of the supernatant fraction obtained folowing the centrifugation of homogenized rat liver at 100,000 x g for 1 hour. Serial dilutions of the supernatant fraction were subjected to SDS-polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol. The proteins were then electrophoretically transferred to nitrocellulose as described in the Materials and Methods section. Following the incubation of the protein (i.e.

Western) blots in anti-PEPCK (1:500 dilution) and anti-RSA (1:2,000 dilution) sera, proteins specifically recognized by the antibodies were visualized using alkaline phosphatase-linked secondary antibody. The secondary antibody consisted of IgG directed against the primary antibody. The results of this experiment are shown in Fig. 4-3. In this system, the antibodies specific for PEPCK and RSA were able to detect their respective target proteins in a minimum of 48 ng of rat liver protein. The utilization of the above-mentioned dilutions for primary and secondary antibodies was consistent throughout the course of this investigation.

Synthesis of PEPCK and RSA in Oocytes

Initial demonstration of PEPCK and RSA synthesis in oocytes

The results of preliminary experiments verifying the rat liver mRNA-induced synthesis of PEPCK and RSA in oocytes are shown in Figs. 4-4 and 4-5. For PEPCK, oocytes were injected with glucagon-treated rat liver mRNA and incubated in MBM at 20°C. After 48 hours, the oocytes were homogenized in OHB and yolk platelets removed via centrifugation. Aliquots of the supernatant fraction were subjected to SDS-polyacrylamide gel electrophoresis and the proteins transferred to nitrocellulose. The Western blots were then examined by means of immunoblot analysis using PEPCK antiserum as described in the Materials and Methods section. Absence of an immunoreactive protein in uninjected oocytes is evident (Fig. 4-4, Immune). However, following the

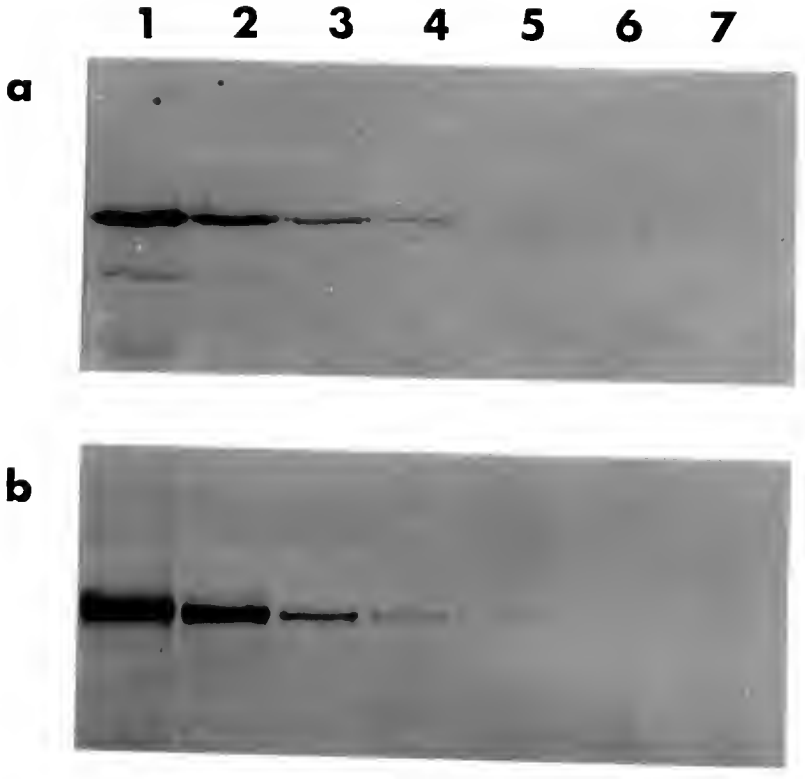

Figure 4-3. Immunoblot analysis of rat liver proteins using PEPCK and RSA antisera. One rat liver from a glucagon-treated animal was homogenized in Tris-buffered 250 mM sucrose and the homogenate centrifuged at 100,000 x g for 1 hour. Serial dilutions of the supernatant fraction were then subjected to SDS-polyacrylamide gel electrophoresis in a 7.5% (w/v) reducing gel. The quantity of protein loaded into each well, in nanograms, is as follows: lane 1 = 30,000; lane 2 = 6,000; lane 3 = 1,200; lane 4 = 240; lane 5 = 48; lane 6 = 9.6; lane 7 = 1.9. The proteins were electrophoretically transferred to nitrocellulose and probed with PEPCK (a) or RSA (b) antisera. The PEPCK and RSA antisera were used at dilutions of 1:500 and 1:2,000, respectively. Proteins specifically bound by the antibodies were identified using alkaline phosphatase-conjugated secondary antibodies as described in the text.

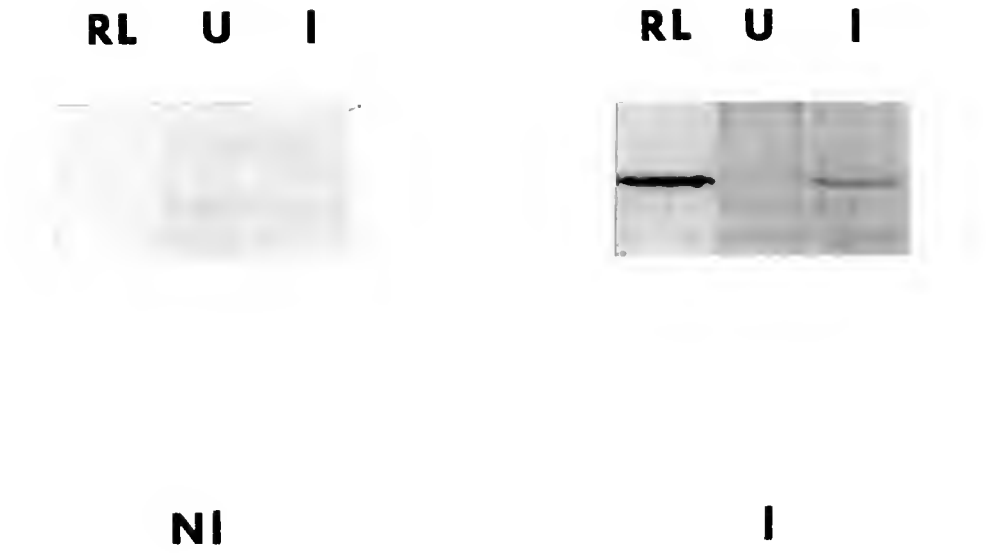

Figure 4-4. Rat liver mRNA-induced synthesis of PEPCK in oocytes. Oocytes were microinjected with poly(A)$^+$ mRNA (35 ng) prepared from the liver of a glucagon-treated rat and incubated in MBM at 20°C for 48 hours. The oocytes were then homogenized in OHB (30 μl/oocyte) and the homogenate centrifuged at 12,000 x g for 10 minutes as described in the Materials and Methods section. Proteins contained in the supernatant fraction were separated via SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with non-immune (NI) or immune (I) anti-PEPCK sheep serum. Identification of proteins specifically bound by the anti-PEPCK antibody was accomplished by means of alkaline phosphatase-conjugated secondary antibody. The total non-yolk protein (approximately 50 μg) from 2 oocytes was loaded into each well of the polyacrylamide gel. (Abbreviations: RL = total rat liver cytosolic protein; U = uninjected oocyte protein; I = injected oocyte protein.)

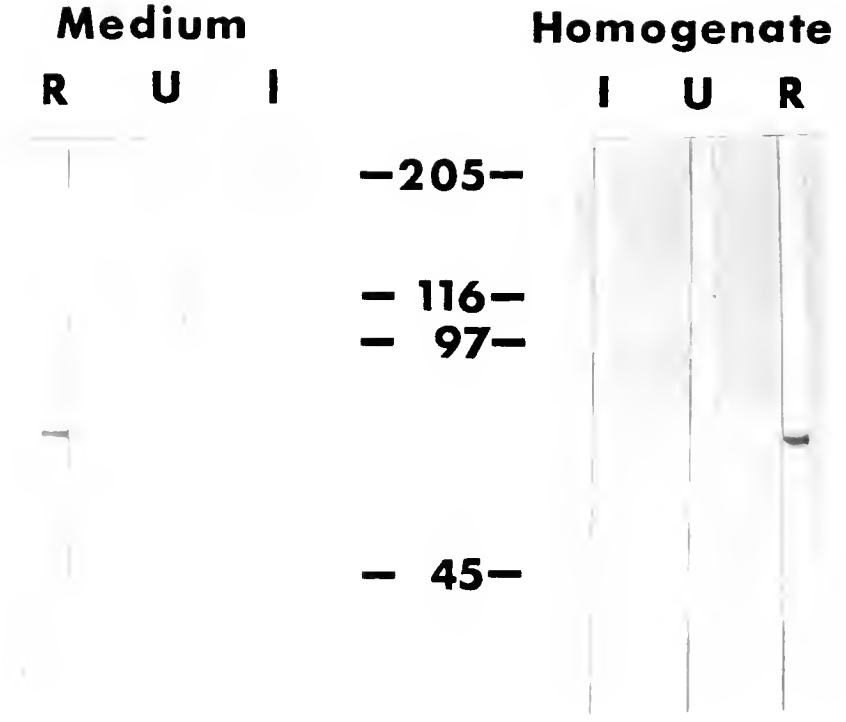

Figure 4-5. Rat liver mRNA-induced synthesis of RSA in oocytes. Oocytes were microinjected with poly(A)$^+$ mRNA (80 ng) prepared from the liver of a fed rat and incubated in MBM at 20°C for 24 hours. Undamaged oocytes were then transferred to fresh MBM at a ratio of 1 oocyte/30 μl MBM and the incubation continued for an additional 24 hours. The medium was then collected and the oocytes homogenized in OHB (30 μl/oocyte). Following centrifugation of the homogenate at 12,000 x g for 10 minutes, the proteins contained in the supernatant fraction or the cell incubation medium were separated via SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with non-immune (not shown) or immune goat serum. Identification of proteins specifically bound by the anti-RSA antibody was accomplished by means of alkaline phosphatase-conjugated secondary antibody. No immunoreactive proteins were observed when non-immune serum was used. The total non-yolk protein (approximately 25 μg) from 1 oocyte was loaded into each well of the polyacrylamide gel. (Abbreviations: R = RSA standard, obtained commercially; U = uninjected oocyte protein; I = injected oocyte protein.)

microinjection of rat liver mRNA, a distinct band (Immune) is observed which migrates at the same molecular weight (69 kD) as that seen in total rat liver protein (Immune). The authentic molecular weight of PEPCK, as determined by amino acid sequence data, is 69.3 kD (Beale et al., 1985).

For RSA, conditions were essentially the same as those just described for PEPCK except that the microinjected mRNA had been isolated from a fed rat. In addition, proteins secreted by the oocytes into the incubation medium were examined as well. From Fig. 4-5 it is apparent that an immunoreactive protein with a molecular weight equivalent to that of authentic RSA (Medium and Homogenate, lane R) is absent from uninjected oocyte homogenate (Homogenate, lane U) as well as medium (Medium, lane U). However, following the microinjection of rat liver mRNA, a band is seen at the expected molecular weight (70 to 72 kDa in a 7.5% (w/v) polyacrylamide SDS gel) in both the oocyte medium and homogenate (Medium and Homogenate, lane I).

Having established the rat liver mRNA-induced synthesis of PEPCK and RSA in oocytes, it was then necessary to define the conditions which would support the production of the greatest amount of these proteins. As stated in the introduction to this chapter, one of the chief goals of my thesis project was to demonstrate the synthesis in oocytes of a rat liver amino acid transporter; specifically, System A. Therefore, the synthesis of PEPCK and RSA was studied in

greater detail in order to serve as a guideline for the synthesis of the System A carrier.

The effect of incubation period on the synthesis of PEPCK and RSA in oocytes

The results of experiments investigating the time course of expression of PEPCK and RSA in oocytes are shown in Figs. 4-6 and 4-7. Oocytes were microinjected with 30 to 35 ng each of glucagon-treated rat liver mRNA. Groups of 10 to 15 oocytes were then homogenized in OHB after incubating for 24, 48, 72, or 96 hours. Media were also collected at the indicated times. Aliquots of the homogenates and media were then subjected to SDS-polyacrylamide gel electrophoresis and the proteins transferred to nitrocellulose. The blots were stained with amido black and photographed prior to immunoblot analysis with PEPCK and RSA antisera. A photograph of the stained blot is shown in Fig. 4-6a. The heavier band at 110 kD in the 48-hour lanes is most likely due to yolk protein contamination as the remainder of the stained bands appear to be of equal intensity for all incubation times. The blot was then cut in half and the two halves used for immunoblot analysis with PEPCK and RSA antisera, respectively. For this and all other experiments requiring the quantitation of the relative amounts of these two proteins, an ^{125}I-linked secondary antibody was used. A photograph of the resulting autoradiograph after PEPCK and RSA immunoblotting is shown in Fig.4-6b. The exposed areas of the autoradiograph were examined by laser scanning densitometry. The densitometric

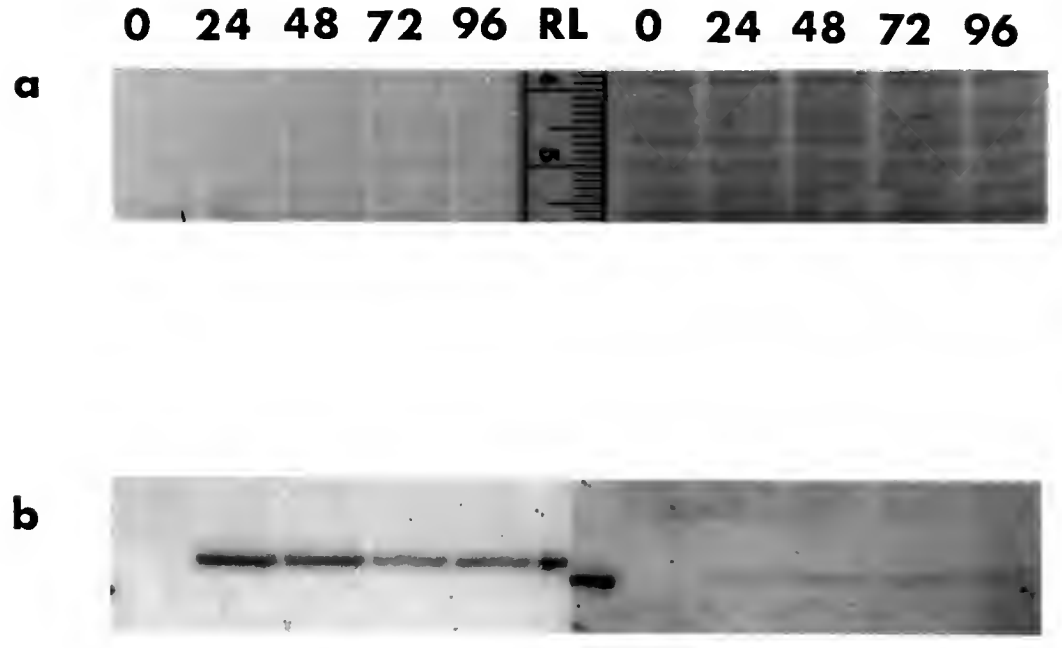

Figure 4-6. Synthesis of PEPCK and RSA in oocytes to 4 days following the microinjection of rat liver mRNA. Oocytes were microinjected with 30 to 35 ng each of glucagon-treated rat liver mRNA and incubated for 0, 24, 48, 72, or 96 hours. The oocytes were then homogenized and the proteins separated via SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and stained with amido black (a). The proteins adhering to the nitrocellulose were then probed with PEPCK or RSA antisera as described in the Materials and Methods section. The secondary antibody was labeled with ^{125}I according to the method of Greenwood et al. (1963). The resulting autoradiograph illustrating the immunoreactive polypeptide is shown in panel b.
Abbreviations: RL = rat liver homogenate.

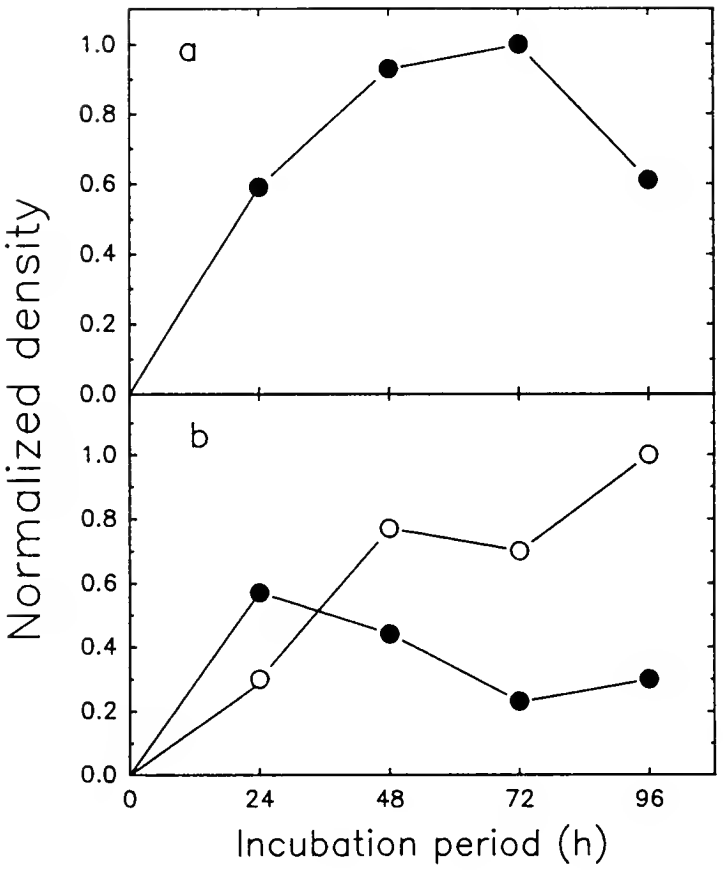

Figure 4-7. Synthesis of PEPCK and RSA in oocytes to 96 hours following the microinjection of rat liver mRNA: results of laser scanning densitometry. Exposed areas of the autoradiograph resulting from immunoreactive proteins (pictured in Fig. 4-6) were quantitated via 2-dimensional laser scanning densitometry as described in the Materials and Methods section. The pattern of PEPCK synthesis is shown in panel a and that for RSA in panel b (closed circles = homogenate; open circles = medium).

data, expressed graphically, are shown in Fig. 4-7. As can be seen in the figure, the amount of PEPCK detected in the oocyte homogenate increases until 72 hours after injection and then declines. Conversely, the quantity of RSA in the oocyte homogenate appears to peak after only one day of incubation while that in the medium continues to rise for the entire 4-day period. The examination of oocytes from numerous experiments has revealed that the viability of injected oocytes declines sharply after approximately 48 hours of incubation. Therefore, the data beyond 48 hours exhibited in Fig. 4-7 may not truly be representative of the time course of foreign mRNA translation in oocytes by may, in actuality, signify their death. For this reason, data concerning the synthesis in oocytes of PEPCK and RSA will be reported only to 48 hours post-injection.

The data in Fig. 4-8 was compiled from 3 separate experiments utilizing oocytes from three different frogs. In all of these experiments, the amount of PEPCK in homogenates of injected oocytes increases during the 48 hours following microinjection (Fig. 4-8, panel a). This is also the case for RSA in the medium and, in two of the three experiments, in the homogenate. There appears, however, to be a lag of greater than 24 hours before the amount of RSA per oocyte in the medium surpasses the amount in the homogenate.

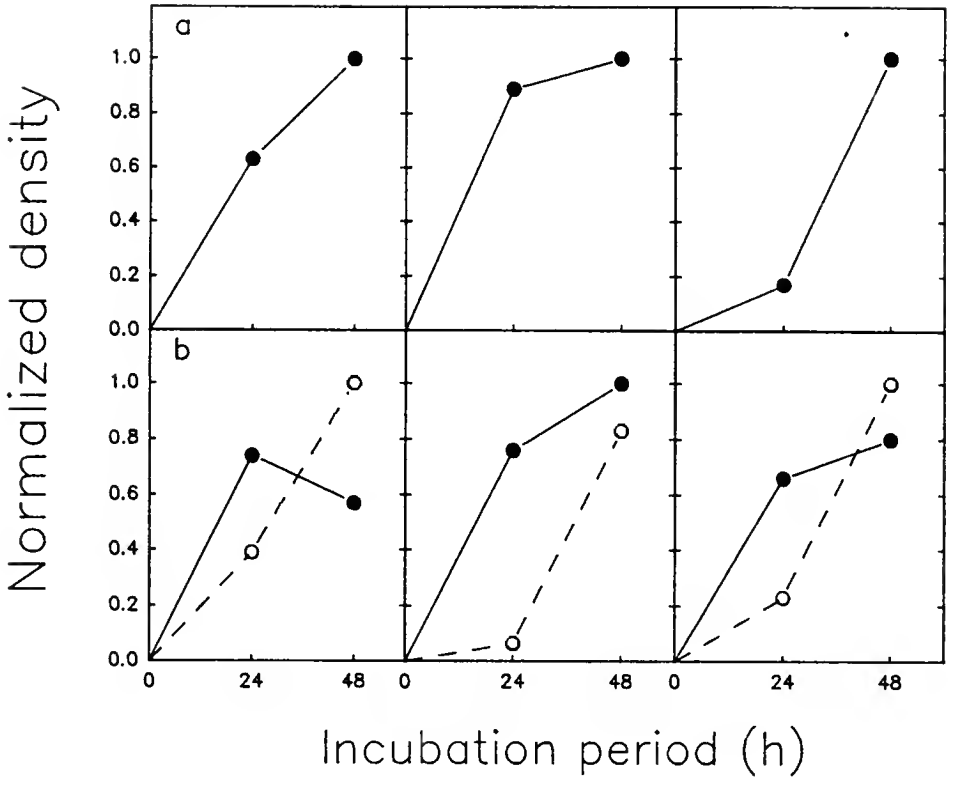

Figure 4-8. Synthesis of PEPCK and RSA in oocytes to 48 hours following the microinjection of rat liver mRNA. Oocytes, obtained from three different frogs, were microinjected with 30 to 35 ng of mRNA from a glucagon-treated rat and incubated for 24 or 48 hours. The oocytes were then homogenized and the proteins separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with PEPCK or RSA antisera. The oocyte incubation media were collected and the proteins contained therein treated in an identical manner. Proteins specifically recognized by the antisera were visualized by means of ^{125}I-labelled secondary antibody and autoradiography. Exposed ares of the autoradiograph resulting from immunoreactive proteins were quantitated by means of 2-dimensional laser scanning densitometry. The results of the densitometric analysis are shown in panel a for PEPCK and in panel b for RSA (closed circles = homogenate; open circles = medium).

The effect of quantity of mRNA microinjected on the synthesis of PEPCK and RSA in oocytes

Following the microinjection of 1, 5, 10, 25, or 50 ng of glucagon-treated rat liver mRNA, PEPCK is detectable in oocyte homogenates after 24 hours of incubation (Fig. 4-9, panel a). Similarly, RSA can be detected in oocyte homogenates as well as media after the same incubation period (Fig. 4-9, panel b). The pattern of protein synthesis in relation to the quantity of mRNA microinjected, however, is different for all three cases. The amount of PEPCK synthesized in oocytes reaches a maximum after the microinjection of 5 or 10 ng of mRNA. The introduction of greater than 10 ng of mRNA results in no further increase in the amount of PEPCK synthesized. In fact, the quantity of this protein may even decrease slightly. The amount of RSA detected in oocyte homogenates, however, rises sharply when the amount of mRNA microinjected is increased from 1 to 5 ng and then continues to increase slowly up to 50 ng. The amount of RSA in the oocyte medium peaks with the injection of 10 ng of mRNA and then is seen to decline with further increases in the quantity of injected message.

Verification of Changes in mRNA Levels by Immunological Analysis of Protein Synthesis in Oocytes

Differences in the relative levels of specific mRNAs in different populations of total RNA can be verified by means of Northern analysis (Fig. 4-2). The ability of the Xenopus oocyte to reproduce these differences by means of mRNA

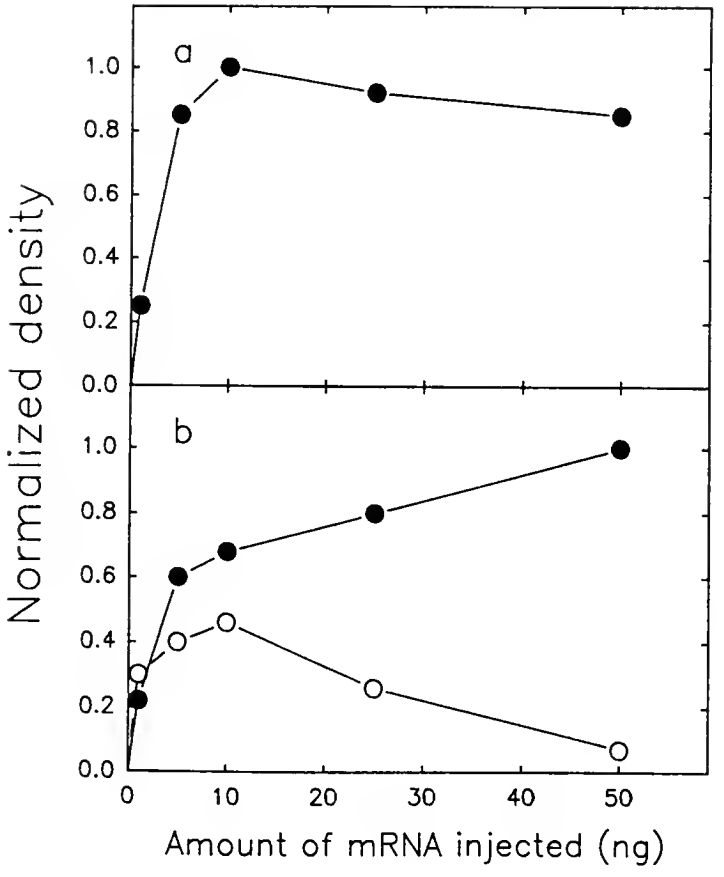

Figure 4-9. Synthesis of PEPCK and RSA in oocytes following the microinjection of rat liver mRNA: effect of mRNA quantity. Oocytes were microinjected with zero to 50 ng of mRNA from a glucagon-treated rat and incubated for 24 or 48 hours. The oocytes were then homogenized and the proteins separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with PEPCK or RSA antisera. The oocyte incubation media were collected and the proteins contained therein treated in an identical manner. Proteins specifically recognized by the antisera were visualized by means of ^{125}I-labelled secondary antibody and autoradiography. Exposed ares of the autoradiograph resulting from immunoreactive proteins were quantitated by means of 2-dimensional laser scanning densitometry. The results of the densitometric analysis are shown in panel a for PEPCK and in panel b for RSA (closed circles = homogenate; open circles = medium).

translation was tested by microinjecting into oocytes total RNA extracted from the livers of fed (i.e., control), diabetic, or glucagon-treated rats. After a 24-hour incubation, oocyte proteins were subjected to immunological analysis using PEPCK and RSA anti-sera as described previously. The results are shown in Fig. 4-10. In this experiment, streptozotocin-induced diabetes caused an 18-fold increase in the quantity of PEPCK mRNA in rat liver when compared to control. Glucagon injection, on the other hand, resulted in a 6-fold increase. In this same experiment, the relative quantity of RSA mRNA was found to have changed very little as a result of diabetes whereas following glucagon treatment it increased over 6-fold when compared to that extracted from the fed rat. Following the microinjection into oocytes of these RNAs, immunoblot analysis demonstrates a pattern of protein synthesis in the oocytes which parallel, in general, the trends in mRNA levels as revealed by Northern analysis (Fig. 4-10). The quantity of PEPCK synthesized in oocytes after the microinjection of diabetic and glucagon-treated RNA was found to be 3- and 1.6-fold greater, respectively, than that synthesized in the oocytes receiving control RNA. Likewise, the largest amount of RSA was found in the homogenates from oocytes microinjected with glucagon-treated RNA while the amount in the oocytes microinjected with diabetic RNA was about two-thirds of the amount in the control oocytes. This duplication of Northern analysis data

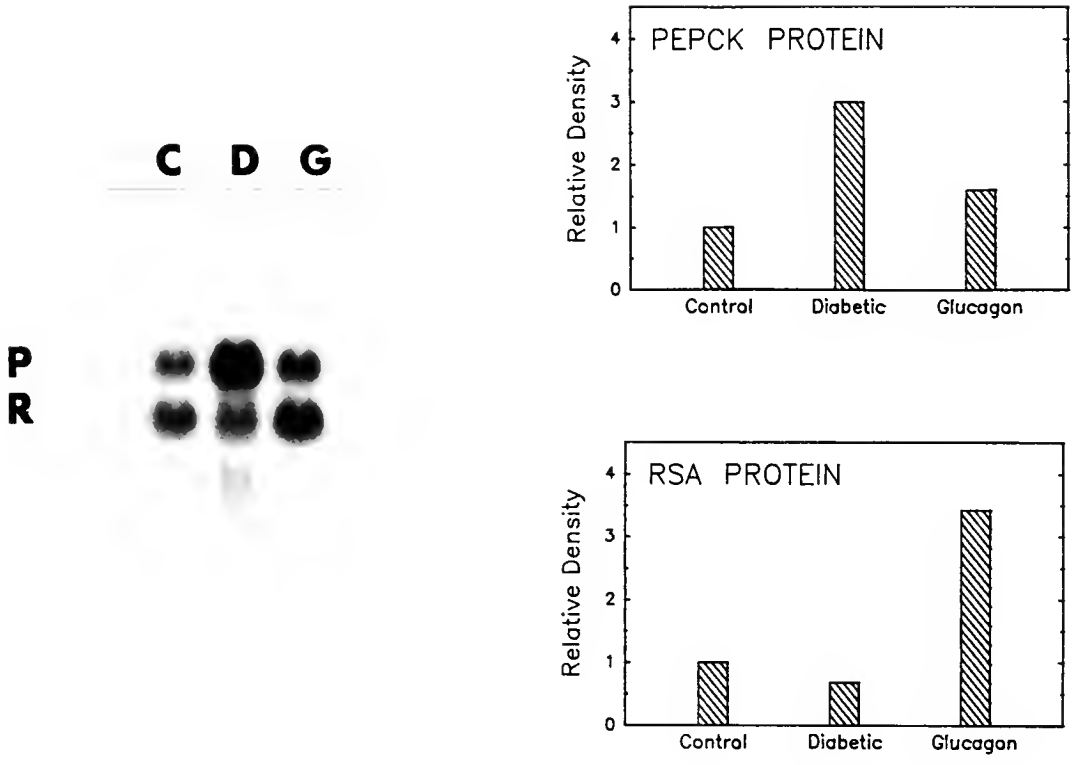

Figure 4-10. Northern and immunoblot analyses of rat liver RNA. Samples (12 μg) of total RNA extracted from the livers of control (C), diabetic (D), and glucagon-treated (G) rats were diluted in RNA loading buffer containing formaldehyde and subjected to electrophoresis in a denaturing 1% (w/v) agarose gel. The gel was photographed under ultraviolet illumination to reveal the ethidium-bromide staining and the resulting negative used for laser scanning densitometry to evaluate RNA loading as described previously. The RNA contained in the gel was then subjected to Northern analysis using ^{32}P-labelled cDNA probes for PEPCK (P) or RSA (R) as described in the legend to Fig. 4-2. Oocytes were microinjected with 220 ng each of total RNA from control, diabetic, and glucagon-treated rat liver and incubated in MBM at 20°C for 24 hours. The oocytes were then homogenized in OHB (30 μl/oocyte) and the proteins contained in the homogenate probed with PEPCK or RSA anti-sera as described in the legend to Fig. 4-4. Identification of proteins specifically bound by the anti-PEPCK or anti-RSA antibodies was accomplished by means of ^{125}I-conjugated secondary antibody. The results of laser scanning densitometry of the resulting autoradiographs are shown in the accompanying bar graphs.

by means of mRNA translation in oocytes was repeated using RNAs extracted from a second group of rats. Although the magnitude of the alterations in mRNA and protein levels was not the same in the two experiments, the trends were essentially identical. In this experiment, diabetes and the injection of glucagon resulted in 7.5- and 2-fold increases, respectively, in the quantity of PEPCK mRNA. The quantity of RSA mRNA was found to have increased by 2-fold in the glucagon-treated rat and to have remained essentially unchanged in the diabetic rat. The microinjection of these RNAs into oocytes followed by the immunological analysis of oocyte proteins reveals a duplication of this pattern. Following the microinjection of RNA extracted from the diabetic rat, the quantity of PEPCK synthesized in oocytes was found to be nearly 4-fold higher than that in the oocytes microinjected with control RNA. The quantity of this protein synthesized by oocytes receiving the glucagon-treated RNA was increased by nearly 3-fold while the amount of RSA was increased 2-fold. Microinjection of RNA extracted from the liver of the diabetic animal resulted in the production of only about one-half of the amount of RSA in the control oocytes.

Degradation of PEPCK and RSA mRNA in Oocytes

As mentioned in the introduction to this chapter, rat liver PEPCK mRNA has a half-life *in vivo* of approximately 40 minutes (Cimbala *et al.*, 1982; Nelson *et al.*, 1980). In

contrast to this relatively rapid turnover rate, the half-life of RSA mRNA can be estimated to be between 14 and 24 hours (Jefferson et al., 1983; Liao et al., 1986; Nawa et al., 1986). In an effort to gauge the degradation rate in oocytes of these two mRNA species, total RNA was extracted from oocytes at various times following the microinjection of rat liver mRNA. This RNA was then subjected to agarose gel electrophoresis, transferred to GeneScreen, and hybridized with ^{32}P-labelled probes for PEPCK and RSA. The results of these experiments are reported in Fig. 4-11. In both of these experiments, the levels of PEPCK and RSA mRNA were seen to decline at fairly constant, and equivalent, rates over the course of four days. The average half-life calculated from the regression lines of both mRNA species in both experiments was found to be 74 ± 3 hours.

A preliminary experiment had demonstrated that the mouse ß-actin cDNA could be used as a probe for Xenopus laevis actin mRNA. An experiment utilizing this probe to investigate RNA that had been extracted from uninjected oocytes incubated over a 6-day period revealed no decrease in the relative amount of oocyte actin mRNA over the course of the incubation (data not shown). These data support the contention that the degradation of PEPCK and RSA in oocytes is not simply a reflection of global oocyte RNA degradation, but a specific response of the oocyte to mRNA species not normally part of the spectrum of endogenous oocyte messages.

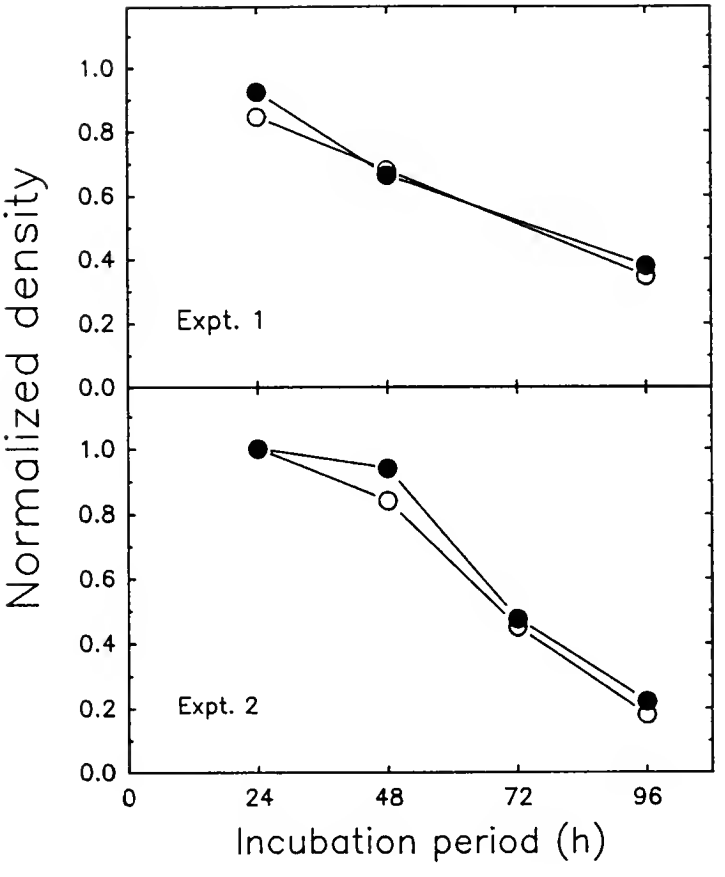

Figure 4-11. Degradation of mRNA for rat liver PEPCK and RSA in oocytes. Rat liver mRNA was microinjected into oocytes which were then incubated in MBM at 20°C for varying amounts of time. At the times indicated in the figure, total RNA was extracted from the oocytes and separated via electrophoresis in a 1% (w/v) agarose gel containing formaldehyde. The RNA contained in the gel was transferred to GeneScreen and hybridized with ^{32}P-labelled probes for PEPCK (open circles) and RSA (closed circles). Hybridization was allowed to continue for 20 to 24 hours after which time the blots were washed in 0.5x (v/v) SSC containing 0.1% (w/v) SDS as described in the Materials and Methods section. The blots were then allowed to expose X-ray film at room temperature or -70°C depending on the amount of radioactivity present. Exposed areas of the autoradiographs were quantitated via 2-dimensional laser scanning densitometry.

Discussion

The preceeding chapter has demonstrated the synthesis in oocytes of rat liver proteins following the microinjection of mRNA. The oocyte-mediated production of two of these proteins, PEPCK and RSA, was followed by means of immunoblot analysis. Relative levels of the mRNAs coding for PEPCK and RSA were determined prior to microinjection by means of Northern analysis.

The regulation of PEPCK mRNA and protein levels in rat liver has been the subject of numerous investigations over the past several years (Hopgood et al., 1973; Tilghman et al., 1974; Gunn et al., 1975). Early work involving changes in PEPCK mRNA levels resulting from various experimental manipulations was based on the ability of mRNA extracted from rat liver to support the in vitro synthesis of the PEPCK protein (Tilghman et al., 1974). The isolation of a cDNA clone of rat liver PEPCK made possible the direct measurement, via hybridization analysis, of PEPCK mRNA levels (Yoo-Warren et al., 1981). Increases in the level of PEPCK mRNA of 8- to 10-fold following the administration of glucagon or dibutyryl cAMP, or the experimental induction of diabetes, have been reported (Beale et al., 1984). This augmentation in the level of PEPCK mRNA in rat liver subsequent to the intraperitoneal injection of glucagon or the induction of diabetes with streptozotocin has been verified in our laboratory (Table 4-1; Fig. 4-10).

Furthermore, an increase in the level of RSA mRNA following glucagon treatment has also been demonstrated (Fig. 4-10). In contrast, diabetes was shown to have little effect on RSA mRNA levels.

As described earlier in this chapter, it is apparent that many factors are responsible for the *in vivo* regulation of albumin gene transcription. Although the quantity of albumin secreted by isolated rat parenchymal cells has been shown to decrease following the administration of glucagon (Dich and Gluud, 1976), the actual level of RSA mRNA was not measured. Nawa *et al*. (1986) have shown that glucagon, in fact, has no detectable effect on the rate of albumin gene transcription *in vitro*. Research in other laboratories has shown that, after a 24- to 30-hour fast, albumin mRNA *in vivo* undergoes a rapid relocation from the membrane-bound polysomal fraction to one possessing sedimentation characteristics of ribonucleoprotein particles (Yap *et al*., 1978). While the actual quantity of albumin mRNA was shown to decline by about 40%, the percentage found on membrane-bound polysomes decreased by nearly 70%. Although this investigation was carried out in the intact rat, the reduction in albumin secretion seen following glucagon administration *in vitro* (Dich and Gluud, 1976) could have been the result of a similar relocation. An inhibition in the rate of albumin gene transcription would, therefore, not be necessary for the synthesis rate of the protein to

decline. Interpretation of the situation *in vivo* is complicated by the fact that the circulating levels of all other hormones, growth factors, and metabolites can never be known. For example, Nawa *et al*. (1986) have shown that, although glucagon has no effect on the transcription rate of the albumin gene *in vitro*, it does modulate the augmentation observed after the application of dexamethasone. Thus, an alteration in the level of albumin mRNA in rat liver following the administration of glucagon will likely be the result of interactions among several messengers as opposed to a direct effect of the hormone. The identification of a glucocorticoid regulatory region 5' to the start of the albumin gene is in agreement with the *in vitro* transcription data and supports the view that the regulation of serum albumin metabolism is controlled primarily by glucocorticoids *in vivo* (Nawa *et al*., 1986). In the experiments discussed in this chapter, glucagon was administered to the animal after a 24-hour fast. Given that the secretion of hormones from the adrenal cortex increases in response to a stress such as starvation (Brown, 1978), the elevation in RSA mRNA levels seen after glucagon administration is not surprising. Although starvation alone may not result in increased albumin mRNA levels, the combined effect of starvation (with the associated elevation in glucocorticoid levels) and artificially high glucagon levels

could result in an augmentation in the rate of albumin gene transcription.

Following the microinjection of rat liver mRNA, PEPCK was detected in oocyte homogenates and RSA was detected in homogenates as well as in the surrounding media. The amount of these proteins synthesized by the oocyte was shown to depend on the length of the post-microinjection incubation period, the quantity of the mRNA injected, and the metabolic state of the animal from which the mRNA was extracted. Both proteins were detected after 24 hours of incubation. In most cases, the amount detected after 48 hours of incubation was greater than that seen after only 24 hours (Fig. 4-7). Continued incubation, however, generally resulted in a decline in the amount of both proteins recovered from the oocyte.

Northern analysis of RNA extracted from oocytes after the microinjection of mRNA has indicated that PEPCK and RSA mRNAs are relatively stable when compared to their half-lives *in vivo*. PEPCK mRNA has been shown to be turned over very rapidly in rat liver and the half-life has been demonstrated to be approximately 40 minutes (Nelson *et al.*, 1980). RSA, on the other hand, is turned over much more slowly. Its half-life has been estimated to be greater than 14 hours (Jefferson *et al.*, 1983; Liao *et al.*, 1986; Nawa *et al.*, 1986). Once inside the oocyte, however, both mRNAs are degraded with an apparent half-life of approximately three

days (Fig. 4-12). The stability of foreign mRNA in oocytes has been investigated in other laboratories and shown to depend upon the presence of a poly(A) tail as well as a 5' cap (Marbaix et al., 1975; Drummond et al., 1985). Because the mRNA used in the experiments described in this chapter was transcribed in vivo (i.e., in rat liver), the repercussions associated with the presence or absence of polyadenylation or a cap structure may be of less importance. In addition, the in vitro translation of this mRNA prior to its microinjection has shown it to be largely intact. Failure to be translated in the in vitro translation system could signify degradation of the mRNA in general. The production of proteins of over 100 kDa MW by in vitro translation verifies the integrity of the mRNA.

Investigations into the metabolism of endogenous Xenopus laevis oocyte mRNA have demonstrated a remarkable stability. Ford et al. (1977) injected [^{3}H]uridine into immature frogs and measured the amount of radioactivity present in oocyte RNA over the next 5 years. The reincorporation of [^{3}H]uridine was investigated by measuring the transfer of radioactivity from RNAs made early in oogenesis (4S and 5S) to those made later (18S and 28S). After correcting for this reincorporation, the authors estimated the half-life of total oocyte mRNA to be at least two years (Ford et al., 1977). In 1979, Darnbrough and Ford, using [^{3}H]ATP, reported that the majority of poly(A) synthesis in mature oocytes is

due to chain extension and turnover rather than de novo synthesis. Thus, it appears that the half-life of 74 hours calculated for PEPCK and RSA mRNA in oocytes is not a reflection of the turnover rate of endogenous mRNA. What factors do govern the half-life of exogenous mRNA, however, are still not known.

Experiments involving the microinjection of varying amounts of mRNA (Fig. 4-9) demonstrate that elevated concentrations of exogenous transcripts in oocytes do not necessarily translate to increased protein synthesis. Although Richter and Smith (1981) have reported that globin synthesis in oocytes rises when the quantity of mRNA microinjected is increased up to 100 ng/oocyte, we find that PEPCK synthesis reaches a maximum after only 10 ng have been microinjected. In the investigation carried out by Richter and Smith (1981), a purified mRNA was microinjected. In the experiment depicted in Fig. 4-9 of this chapter, total rat liver mRNA was used. Therefore, the possibility exists that the discrepancy in saturating concentrations of mRNA is the result of these vastly different mRNA populations. At best, PEPCK represents approximately 1% of rat liver mRNA (Meisner et al., 1983). Other less abundant mRNA molecules may be able to compete more effectively for ribosome binding and, hence, cause oocyte-mediated PEPCK synthesis to saturate at low levels of microinjected mRNA.

In the experiment described in Fig. 4-10, total RNA, as opposed to poly(A)$^+$ mRNA, was microinjected into oocytes. The RNA had been extracted from the livers of fed, diabetic, or glucagon-treated rats. Northern analysis, performed on samples of these same mRNA populations, revealed increases in the levels of PEPCK mRNA following the induction of diabetes or the injection of glucagon. RSA mRNA levels were shown to be elevated in the glucagon-treated rat and slightly depressed in that of the diabetic rat. In an effort to preserve the relative levels of these mRNAs, oligo(dT) cellulose column chromatography was not performed. Agarose gel electrophoresis of poly(A)-selected mRNA had previously shown that variable amounts of ribosomal RNA remain even after two passes over the oligo(dT) column. Retention of the levels of PEPCK and RSA mRNA as revealed by Northern analysis would not have been possible had poly(A)$^+$ mRNA selection been attempted. Unless the precise amount of ribosomal RNA remaining in the mRNA sample is known, accurate quantitation of poly(A)$^+$ mRNA is not possible.

The results of the immunoblot analyses represented in Fig. 4-10 display a trend in protein synthesis equivalent to that seen in mRNA level. We believe this to be the first demonstration of an oocyte-translation based duplication of Northern analysis for a transcriptionally-regulated gene.

Given the data regarding PEPCK and RSA synthesis in oocytes, preliminary experiments investigating the oocyte-

mediated production of the rat liver System A carrier were carried out. In two separate experiments, increased rates of Na^+-dependent AIB transport were detected in oocytes following the microinjection of exogenous mRNA. In one experiment, the rate of Na^+-dependent AIB transport increased from 4.4 ± 1.5 pmol·oocyte^{-1}·h^{-1} to 7.5 ± 1.5 pmol·oocyte^{-1}·h^{-1} after 24 hours of incubation. In this experiment, control oocytes were microinjected with HEPES buffer and the experimental oocytes with the same buffer containing mRNA extracted from the liver of a glucagon-treated rat. In a second experiment, the oocytes were microinjected with mRNA that had been extracted from wild type CHO-K1 cells or from the mutant ala^{r}4-H3.9 CHO-K1 cells exhibiting elevated System A activity (Moffett et al., 1983; Moffett et al., 1988). After 51 hours of post-microinjection incubation, the oocytes receiving the wild-type mRNA displayed a Na^+-dependent AIB transport rate of 2.8 ± 2.0 pmol·oocyte^{-1}·h^{-1} while those receiving the mutant mRNA displayed a rate of 10.5 ± 5.0 pmol·oocyte^{-1}·h^{-1}. Although the standard deviations are rather large, as is often the case with oocytes, the experimental values were significantly different from the control values ($P<0.005$ for the first experiment and $P<0.01$ for the second experiment).

In spite of the fact that increases in Na^+-dependent AIB transport were observed following the microinjection of exogenous mRNA, consistent results between experiments

proved to be elusive. For example, a repeat of the second experiment mentioned above resulted in no significant increase in System A activity even though a 3.8-fold increase was seen initially. From the investigations of PEPCK and RSA synthesis in oocytes, it is clear that large differences in the amounts of proteins produced in the oocyte resulting from a given population of mRNA may be encountered. Also, large differences in the relative abundance of various mRNAs is not uncommon. Even after the induction of diabetes in rats, which causes an impressive elevation in PEPCK mRNA levels, this transcript represents only about 1% of the total liver mRNA (Meisner et al., 1977). RSA mRNA, on the other hand, represents approximately 10% of the total rat liver mRNA (Feldhoff et al., 1977). These differences are reflected in the amount of the two proteins synthesized in oocytes following the microinjection of rat liver mRNA. Based on the quantity of oocyte protein loaded onto polyacrylamide gels and the intensity of exposed areas on autoradiographs following ^{125}I-immunoblot analyses, it appears that 20 to 30 times as much RSA is produced in the oocyte as compared to PEPCK. If we assume that the abundance of the mRNA coding for the System A transporter is equal to or less than that for PEPCK, the amount of carrier protein inserted into the oocyte plasma membrane may be exceedingly difficult to detect by the assay methods currently available. This is supported by experiments identical to those described above, but in

which no increase in System A activity was observed. Development of other assays, more sensitive than accumulation of amino acid, may solve this dilemma. Among these could be included electrophysiologic measurements of amino acid-dependent Na^+ movement.

In summary, the oocyte-mediated synthesis of two rat liver proteins, PEPCK and RSA, has been demonstrated. Evidence for the synthesis of a third protein, the System A amino acid transporter, has been presented also. In addition, alterations in the relative quantities of rat liver PEPCK and RSA mRNA following the induction of diabetes with streptozotocin or the injection of glucagon has been documented via Northern analysis and shown to be reflected accurately by translation of hepatic mRNA in Xenopus oocytes.

CHAPTER V

CONCLUSIONS AND FURTHER DIRECTIONS

The uptake of amino acids by manually-defolliculated oocytes has been shown to be mediated by several distinct transport activities. In some instances, uptake characteristics for a particular amino acid or group of amino acids resembled those previously reported for transport systems in other tissues. For example, the Na^+-independent transport of arginine was inhibited over 80% by the inclusion of cationic amino acids in the uptake mixture. Neutral and negatively-charged amino acids, however, displayed little inhibitory activity (Fig. 2-5). These characteristics are very similar to those described for System y^+ in human fibroblasts (White, 1982). Furthermore, the Na^+-dependent portion of arginine transport in oocytes appears to be mediated, in part, by a carrier with specificity for both neutral and cationic amino acids (Fig. 2-3). A transport system exhibiting this broad substrate specificity was described in 1985 by Van Winkle et al. and has been named System $B^{o,+}$.

System $B^{o,+}$, initially characterized in mouse blastocysts, is believed to function in the activation and uterine wall attachment of metabolically quiescent embryos

(Van Winkle et al., 1985). The period of quiescence prior to activation, termed diapause, lasts throughout the time the previous litter is nursed and ceases at weaning. The ensuing release from diapause appears to be dependent upon the presence of sodium as in vitro activation can be prevented by culturing blastocysts in medium containing low levels of this ion (Van Winkle et al., 1985). Accumulation of amino acids, via System $B^{o,+}$, during activation is thought to be necessary for completion of the process. Given that amino acid transport through System $B^{o,+}$ is Na^+-dependent, it could be that the action of this carrier is responsible for at least a portion of the sodium requirement seen in activation.

Even though the characteristics of Na^+-dependent arginine transport in oocytes resemble those of System $B^{o,+}$, it can not yet be stated with certainty that the oocyte activity is the result of the same carrier protein. In addition, a role for System $B^{o,+}$ in Xenopus oocytes analogous to its role in the mouse is difficult to imagine in two systems as dissimilar as these. Following the completion of oogenesis in Xenopus laevis, mature oocytes can remain arrested in meiotic prophase for many months. In the wild, ovulation is triggered hormonally in response to environmental cues and fertilization takes place in the water (Deuchar, 1975). As described in Chapter 3, the continuation of meiosis up to metaphase of the second division is accompanied by a cessation of essentially all plasma membrane

amino acid transport activity. Clearly then, if a transporter analogous to the murine system described above does exist in Xenopus laevis, it is more likely to play a role in events taking place after fertilization than before. Although some investigations of amino acid transport in frog embryos have been carried out (Hampel et al., 1975; Mlot et al., 1978), a systematic search for System $B^{o,+}$ had not been attempted previously. The results of the above mentioned characterization of endogenous amino acid transport in oocytes has laid the groundwork for future studies on expression of hepatic carriers.

As mentioned above, the translation of exogenous mRNA in Xenopus laevis oocytes has been demonstrated numerous times since the development of the technique in 1971 (Gurdon et al., 1971; Lane et al., 1971). The investigation summarized in Chapter IV illustrates the oocyte-mediated synthesis of two additional proteins: PEPCK and RSA. Although preliminary evidence has been presented for the synthesis of a third protein, the rat liver System A carrier, unequivocal proof is not possible without protein identification. The data presented in the discussion section of Chapter IV indicate that, although promising, the mRNA-induced synthesis of System A in oocytes requires further optimization before consistent results can be achieved. The results of experiments involving the oocyte-mediated production of PEPCK show that only minute quantities of the protein are

detectable by immunoblot analysis. Whether this is due to a failure of the oocyte system to efficiently translate this mRNA or to an instability of the protein in the oocyte is not known. Based on the sensitivity of the anti-PEPCK antibody used (FIG. 4-3) and examination of autoradiographs of immunoblot analyses, it appears that approximately 1 ng of PEPCK is detectable in each oocyte after a 24-hour incubation. Using an approximate molecular weight of 70 kDa for PEPCK (Beale et al., 1985), this translates to roughly 10^{10} molecules of the enzyme per oocyte. If we assume the same number of System A molecules to be present in the oocyte plasma membrane after mRNA microinjection and if we assume a transit time of 1 AIB molecule through the carrier per second, only 10 cpm of ^{3}H will have accumulated in each oocyte after a 1-hour incubation using the transport assay described in the text. In addition, this 10 cpm must be detected against the background of endogenous AIB transport in the oocyte. Granted, there are several assumptions which have been made in this estimation, any of which could be in error. However, errors are as likely to be in favor of diminished accumulation of radioactively-labelled substrate as they are in the opposite direction. In short, the possibility exists that the detection of rat liver System A in the oocyte may be pushing the sensitivity of the transport assay to its limits.

Besides the difficulty associated with the low abundance of the System A protein, there is also the question of

functionality. Although all of the exogenous channels and receptors reported to have been synthesized in the oocyte appear to retain their native activity (Parker et al., 1985), it is not known if the System A carrier is, in fact, in a functional state when inserted in the oocyte plasma membrane. Further, a question which goes beyond that of structural maladies concerns the phenomenon of trans-inhibition. One of the identifying characteristics of System A, whether measured in intact cells or plasma membrane vesicles, is its sensitivity to the presence of substrate amino acids on the side of the membrane opposite to that from which transport is being measured (i.e., the "trans" side). This phenomenon is referred to as trans-inhibition. It has been reported that the concentration of small, neutral amino acids (i.e., System A substrates) in mature oocytes is approximately 3 mM (Bravo et al., 1976). If this is the case, the activity of rat liver System A in oocytes could be depressed. Because incubation in amino acid-free medium does not deplete this endogenous store (Bravo et al., 1976), all transport measurements are, therefore, performed in the face of this intra-oocyte amino acid concentration. A possible solution is to measure amino acid transport rates using oocyte plasma membrane vesicles instead of intact cells. Vesicles formed in the absence of amino acids would, by definition, not have System A compromised by trans-inhibition.

Still another concern which must be attended to with regard to the measurement of amino acid transport rates in oocytes subsequent to microinjection is leakage. Based on experiments performed by Miller et al. (1984), it appears that damage done to the amphibian oocyte by microinjection may be, to an extent, irreversible. Although these experiments were performed using Rana pipiens oocytes, the possibility that similar perturbations in the ionic content of Xenopus laevis oocytes subsequent to microinjection could not be ruled out. The results of this investigation demonstrated that oocytes incubated in Ringer's solution following microinjection tended to lose potassium from the cytoplasm while gaining sodium from the surrounding medium. Within 18 hours after microinjection, the intra-oocyte potassium concentration was shown to fall from approximately 120 mM to 40 mM while tha sodium level increases from 50 mM to over 150 mM. Incubation of the oocytes in mineral oil, rather than Ringer's solution, following microinjection has been shown, however, to staunch the flow of ions through the plasma membrane (Miller et al., 1984). An increase in intra-oocyte sodium to a level equivalent to that in the incubation medium would be particularly detrimental to assays involving the measurement of Na^+-dependent amino acid transport rates. Investigations into the impact of incubation in MBM-saturated mineral oil on the Na^+-dependent transport of AIB in Xenopus oocytes have shown no observable protective

effect. Following an overnight incubation in oil, both ddH_2O-injected and uninjected oocytes displayed very low rates of Na^+-dependent AIB transport (0.59 and zero $pmol \cdot oocyte^{-1} \cdot h^{-1}$, respectively) while those incubated in MBM displayed rates typically observed in defolliculated oocytes (1.64 and 1.46 $pmol \cdot oocyte^{-1} \cdot h^{-1}$ for injected and uninjected, respectively). Further, separate experiments involving the microinjection of radiolabelled sucrose and inulin (MW 5,000 Daltons) into Xenopus oocytes revealed no detectable leakage after four hours of incubation in MBM. This is in contrast to the situation in Rana pipiens oocytes in which the loss of injected sucrose proceeds at a rate of approximately 5% per hour (calculated from Miller et al., 1984). Therefore, it appears that Xenopus oocytes may be able to recover more effectively from microinjection than Rana pipiens oocytes.

In summary, regardless of the situation described for the System A amino acid transporter in particular, the investigation of hepatic PEPCK and RSA synthesis in oocytes and the characterization of endogenous oocyte amino acid transport represents an important contribution to the field in general. In the attempted oocyte expression of proteins for which antibodies and cDNA clones are lacking, it is important to have some idea of the time-course of exogenous protein synthesis as well as the degradation rate of mRNAs that may be handled in a similar manner by the oocyte. By utilizing proteins dissimilar in their regulation and

compartment of synthesis (i.e., PEPCK and RSA), but each having some parallels with transporters, the applicability of the data is broadened. In addition, the mRNAs coding for the two proteins differ in their relative abundance as well as their half-lives in vivo. For both PEPCK and RSA synthesis in oocytes, post-microinjection incubations of 24 to 48 hours have been demonstrated to be sufficient. In addition, the microinjection of large quantities of mRNA appears not to be necessary as lower levels result in the production of very adequate amounts of foreign proteins in oocytes. It has been shown also that concerns regarding the stability of microinjected mRNA may be unfounded as PEPCK mRNA, with a very short in vivo half-life (40 minutes), displayed an intra-oocyte half-life of at least 3 days. Although the mRNAs coding for both PEPCK and RSA displayed half-lives in oocytes of equal magnitude, the possibility exists that transcripts coding for other proteins will be degraded more, or less, rapidly. If this is the case, the Xenopus oocyte system may prove to be very useful in deciphering the signals responsible for mRNA half-life in vivo. Finally, Fig. 4-10 demonstrates that the Xenopus oocyte system can be used to estimate relative mRNA levels provided an antibody or a suitable assay exists for the protein in question. Information regarding the degree of induction of a particular mRNA would be very useful, for example, prior to the production of a cDNA library. The

identification of a particular cDNA within the library could be simplified considerably with the utilization appropriate conditions designed to increase its representation.

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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